A RANDOMIZED PHASE II TRIAL TO EVALUATE PROGRESSION-FREE SURVIVAL RATES IN PATIENTS RECEIVING NK CELL-ENRICHED DONOR CELL INFUSIONS WHEN ADMINISTERED ALONE OR ADMINISTERED WITH THE TLR9 AGONIST, DUK-CPG-001, FROM A 4-6/8 HLA-MATCHED RELATED OR 7-8/8 HLA-MATCHED DONOR FOLLOWING ALLOGENEIC STEM CELL TRANSPLANTATION

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LIST OF ABBREVIATIONS

aGVHD	Acute Graft Versus Host Disease
AE	Adverse Event
ALL	Acute Lymphoblastic Leukemia
ALT	Alanine Aminotransferase
AML	Acute Myeloid Leukemia
API	Active Pharmaceutical Ingredients
AST	Aspartate Aminotransferase
BUN	Blood Urea Nitrogen
CAP	College of American Pathologists
CBC	Complete Blood Count
CIBMTR	Center for International Bone and Marrow Transplant Research
CLIA	Clinical Laboratory Improvement Amendments
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myelogenous Leukemia
CMV	Cytomegalovirus
CPC	Cancer Protocol Committee
CpG	Unmethylated deoxycytidyl-deoxyguanosine
CR	Complete Response
CRF	Case Report Form
CT	Computed Tomography
CTCAE	Common Terminology Criteria for Adverse Events
CTQA	Clinical Trials Quality Assurance
DCI	Duke Cancer Institute
DLI	Donor Lymphocyte Infusion
DSMB	Data and Safety Monitoring Board
DUHS	Duke University Health System
ECOG	Eastern Cooperative Oncology Group
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GI	Gastrointestinal
GMP	Good Manufacturing Practice
GVT	Graft versus Tumor
HLA	Human Leukocyte Antigen
HPCA	Hematopoietic Progenitor Cell Assay
HRPP	Human Research Protections Program
HSV	Herpes Simplex Virus
ICH	International Conference on Harmonization
ICS	Investigational Chemotherapy Service
IND	Investigational New Drug
IRB	Institutional Review Board
IV (or iv)	Intravenously
LDH	Lactate Dehydrogenase
MRI	Magnetic Resonance Imaging
MUD	Matched Unrelated Donor
NCI	National Cancer Institute
NHL	Non-Hodgkin Lymphoma
NK	Natural Killer Cell
NMDP	National Marrow Donor Program
ODNs	Oligodeoxynucleotides

OS	Overall Survival
OSHA	Occupational Safety and Health Adminstration
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
pDCs	Plasmacytoid Dendritic Cells
PET	Positron Emission Tomography
PFS	Progression Free Survival
PI	Principal Investigator
PR	Partial Response
RFLP	Restriction Fragment Length Polymorphism
SAE	Serious Adverse Event
SD	Standard Deviation
SLL	Small Lymphocytic Lymphoma
SOC	Safety Oversight Committee
SOP	Standing Operating Procedures
ULN	Upper Limit of Normal
VZV	Varicella Zoster Virus
WBC	White Blood Cells

PROTOCOL SUMMARY

This randomized, parallel phase II study is designed to evaluate the rates of progression-free survival and unacceptable toxicity in patients receiving NK cell-enriched DLIs when administered alone or administered with the TLR9 agonist, DUK-CPG-001, from a 7-8/8 HLA-matched related or unrelated donor (Cohort A) or 4-6/8 HLA-matched related donor (Cohort B) following allogeneic stem cell transplantation.

Objectives

The primary objectives of this study are:

1. To evaluate the one-year progression-free survival rates in patients receiving NK cell-enriched DLI administered alone from a 7-8/8 HLA-matched related or unrelated donor or 4-6/8 HLA-matched related donor following allogeneic stem cell transplantation.

2. To evaluate the one-year progression-free survival rates in patients receiving NK cell-enriched DLI administered with a Toll-like receptor 9 (TLR9) ligand, DUK-CPG-001, from a 7-8/8 HLA-matched related or unrelated donor or 4-6/8 HLA-matched related donor following allogeneic stem cell transplantation

3. To evaluate the rates of unacceptable toxicity of using NK cell-enriched DLI administered alone from a 7-8/8 HLA-matched related or unrelated donor or 4-6/8 HLA-matched related donor following allogeneic stem cell transplantation.

4. To evaluate the rates of unacceptable toxicity of using NK cell-enriched DLI administered with TLR9 ligand, DUK-CPG-001, from a 7-8/8 HLA-matched related or unrelated donor or 4-6/8 HLA-matched related donor following allogeneic stem cell transplantation.

The secondary objectives of this study are:

1. To evaluate the recovery of immune cell populations pre and post infusion in patients receiving NK cell-enriched DLI administered alone or administered with TLR9 ligand, DUK-CPG-001.

2. To evaluate the immune function with antigen specific recovery with Elispot, Immunoscope, and flow-cytometric cytokine assay, and functional NK lysis assay, pre and post infusion in patients receiving NK cell-enriched DLI administered alone or administered with TLR9 ligand, DUK-CPG-001.

Patient population

Patients who have undergone an allogeneic transplantation, using a 7-8/8 HLA-matched related/unrelated donor (Cohort A) or 4-6/8 HLA-matched related donor (Cohort B). Specific inclusion and exclusion criteria are detailed in 3.0 Eligibility criteria.

Number of patients

A total of 50 patients (25 patients in each arm) will be enrolled in Cohort A, and 50 patients (25 patients in each arm) will be enrolled in Cohort B.

Study design and methodology

This is a randomized, parallel phase II study to evaluate the rates of progression-free survival and unacceptable toxicity in patients receiving NK cell-enriched DLIs when administered alone or administered with the TLR9 agonist, DUK-CPG-001, from a 7-8/8 HLA-matched related/unrelated donor (Cohort A) or 4-6/8 HLA-matched related donor (Cohort B) following allogeneic stem cell transplantation. Randomization will be stratified for disease types (myeloid versus lymphoid malignancies). Primary endpoints are analyzed separately in Cohort A and B.

Cohort A: 8/8 HLA-matched (related or unrelated) donor
("NK cell enriched-DLI only" arm or "NK cell enriched-DLI + DUK-CPG-001" arm)

Cohort B: 4-7/8 HLA-matched related donor
("NK cell enriched-DLI only" arm or "NK cell enriched-DLI + DUK-CPG-001" arm)

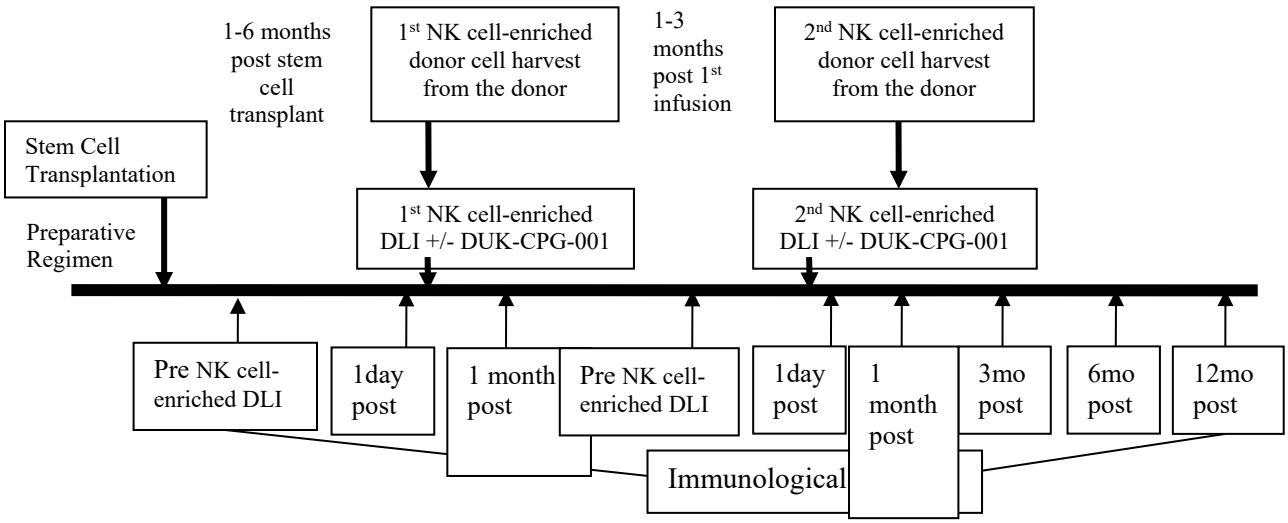

Figure 1 Trial design for NK cell-enriched DLI +/- DUK-CPG-001

1.0 PURPOSE

Allogeneic stem cell transplantation may cure or ameliorate many types of diseases, however the toxicity and lack of suitable donors limits its applicability. Possible solutions to these problems include a less toxic, reduced intensity/non-ablative conditioning regimen and using HLA partially mismatched donors. Our group has shown that a less toxic preparative regimen can reliably allow engraftment with much less risk of complications from the transplant procedure.[1] Further, initial response rates are high. However, long term remissions are lacking. Use of donor lymphocyte infusions (DLIs) in this setting following transplant may allow increased anti-tumor efficacy. DLIs can exert a graft-versus-tumor (GVT) effect and help eliminate residual disease after transplantation. Their use is however, complicated by the risk of acute graft-versus-host disease (aGVHD) with 30-40% of patients experiencing grade III-IV aGVHD. Data suggests that the use of NK cells (instead of nonselected DLIs) in this setting may mediate a GVT effect independently of aGVHD. Our recent pilot study demonstrated that the NK cell-enriched DLI with a 1-step high-yield process is feasible with little toxicity.[2] The NK cell-enriched DLI resulted in improved immune recovery and outcomes for some patients; it suggests that further manipulation or modification of NK cell-enriched DLI would be a promising strategy to obtain better immune recovery and outcomes under the platform of allogeneic stem cell transplantation. This randomized, parallel phase II study is designed to evaluate the rates of progression-free survival and unacceptable toxicity in patients receiving NK cell-enriched DLIs when administered alone or administered with the TLR9 agonist, DUK-CPG-001, from a 7-8/8 HLA-matched related or unrelated donor (Cohort A) or 4-6/8 HLA-matched related donor (Cohort B) following allogeneic stem cell transplantation.

2.0 BACKGROUND

Myeloablative allogeneic stem cell transplantation may cure many types of diseases, but the toxicity associated with it limits its applicability. We and others have shown that reduced intensity/non-ablative therapy allows allogeneic therapy to be used by older, more infirmed patients with various types of neoplastic diseases and marrow failure syndromes.[3,4,5,6] Our results have yielded nearly 100% donor engraftment with approximately a 10% death rate due to the procedure.[7] Early response rates in the 75% range are encouraging in patients with minimal residual disease at the initiation of therapy, however long term remissions are still lacking. Earlier immune recovery may allow increased anti-tumor efficacy.[8] Donor lymphocytes are one mechanism to assist earlier immune recovery and improved anti-tumor efficacy. However, the use of DLIs is limited by the risk of aGVHD. On the other hand, NK cells may mediate a GVT effect independently of aGVHD.

2.1 Graft vs Tumor Effects in Allogeneic Stem Cell Transplantation

Most patients with hematologic malignancies are not cured with standard chemotherapy and novel approaches are needed to improve outcomes. One novel approach is allogeneic hematopoietic stem cell transplantation to induce an immunologic effect against the cancer. In patients with leukemia, this has been termed a graft-versus-leukemia effect. Evidence for this includes:

1. a temporal relationship between GVHD and hematologic remission[9,10]
2. a reduced incidence of leukemic relapse after allogeneic bone marrow transplantation compared to syngeneic bone marrow transplantation[11]
3. a reduced incidence in leukemic relapse in allogeneic transplant recipients who do develop GVHD compared to those who do not[12]

Multiple laboratory studies have been published in support of this clinical data as well. Tumor specific antigens and histocompatibility antigens may play a role and the interactions may be through direct cell contact, activating NK cells, or indirect production of cytotoxic cytokines (interferon, TNF-alpha, IL-2, and IL-12).

2.2 Safety and Efficacy Data With Less Toxic Nonmyeloablative or Reduced-intensity Regimens

The nonmyeloablative and reduced-intensity regimens have been promulgated to provide older, more infirmed patients an opportunity to benefit from graft-versus-tumor effects. In myeloid and myeloproliferative diseases, reduced-intensity transplantation is noted to have about a 15-20% treatment-related mortality with 40% long-term survival.[13] Those with high-risk lymphomatous diseases in general are reported to have a slightly lower early death rate from the procedure (approximately 10%) with 50-60% long-term disease-free survival[14,15] including encouraging data for those with CLL.[16,17] Patients with myeloma or relapsed Hodgkin's disease are noted to possibly benefit as well.[18,19] Data exists with success for every type of high-risk or relapsed hematologic malignancy or myeloproliferative disorder, however relapse and infections remain problematic and much work remains to improve outcomes.

General efficacy for Allogeneic Transplant: Our program has focused our development in this area on T cell depleted grafts to assist in decreasing early GVHD and toxicity concerns. It has been documented by some that T cell depletion in allogeneic transplantation of various types can be associated with increased toxicities of infections and relapse. The Boston group noted in a review of chronic phase CML patients that T depletion led to less early toxicity but increased 3 year relapse rates (62 vs 24%).[20] Recent retrospective reviews by the MD Anderson group comparing T cell depleted grafts to unmanipulated grafts, though, have not been as clear cut for long term negative effects of T depletion. Their data shows overall no significant difference in long term relapse or survivals, but a significant increase in early infectious deaths in the T cell depleted cohorts (5% vs 1% 100 day mortality). In both the T depleted and T replete first remission AML patients the 3 year survivals were still only 57-58%. Relapse accounted for 15% (in T depleted group) and 23% (unmanipulated group) of the deaths and infectious deaths were also common (11 and 26% of the deaths), suggesting improvements in immune reconstitution might be important. [21] Similarly, lymphoma patients may undergo allogeneic transplantation at times as well. Lee and colleagues have recently published a T depleted reduced intensity approach for patients with NHL or Hodgkins lymphoma noting only a 40% 5 year overall survival and 39% progression free survival.[22] Further recent data in relapsed mantle cell patients shows 3 year PFS of only 41%.[23] Recent data by Wang et al showed in a small group of relapsed or refractory patients only approximately a 50% 2 year progression free survival. [24] Additionally, a recent multicenter study reported on nonablative allogeneic transplant for lymphoma patients (various B cell, T cell, or hodgkins) in whom autologous transplantation had already failed. The 3 year PFS was also only in the 44% range with the most common reason for death being relapsed disease.[25] In general, data from the CIBMTR has shown that by far the most common reason for post transplant death is the primary disease (48%) with further contribution from infections (13%) [26], indicating that efforts to improve immune recovery are of paramount importance. In reviewing the CIBMTR data for 27,941 patients undergoing a matched sibling or unrelated donor transplant for AML, the 3 year survival for those in first remission was still only 58% and 49% respectively and those with advanced disease had only 24% and 22% survival. For those with MDS, even in the 'early disease' state the 3year survival was only 53% from a matched sibling and 49% from a MUD (data from 2002-2012). In adult ALL patients, the 3 year survival using a matched donor was only 55% in the 'early disease' and 26% for the advanced disease patients and in the MUD setting 53% and 20%. For Hodgkin disease, the 3 year survival from a matched donor was only 52% and 45% from a MUD. Allogeneic transplant for diffuse large cell lymphoma is typically reserved for those with more aggressive disease and as reported to the CIBMTR 3 year survival is only 50% for those with a matched sibling even with chemosensitive disease. Similarly, for the 1,104 patients with myeloma who have undergone an allogeneic transplant, the 3 year survival is only 50%. [26]

Thus, this study focuses on patients with only about a 50% chance of durable response from the allogeneic transplant at best, with infections and relapse being prime causes of morbidity and/or mortality. These data and others suggest that all patients post allogeneic transplant might be appropriate to consider an immunotherapy of some type except for the very good risk patients such as those with aplastic anemia, CLL or SLL or follicular lymphoma in first remission having needed only 1 regimen to attain a CR, or CML in first chronic phase entering in molecular remission without known molecular mutations.

2.3 Our program's Recent Preliminary Results Utilizing Fludarabine, Melphalan or Busulfan, and Alemtuzumab for Nonmyeloablative Therapy

We are currently conducting nonmyeloablative transplantation using fludarabine (160 mg/m2), melphalan (140 mg/m2), and alemtuzumab (80mg) for patients with lymphoid or myelomatous diseases, and fludarabine (160 mg/m2), busulfan (260 mg/m2), and alemtuzumab (80 mg) for patients with myeloid diseases.[27] Preliminary analysis of 98 patients showed that 1-year progression-free survival for 6/6 HLA-matched related or unrelated donor (n = 69) and 3-5/6 HLA-matched related donor (n = 29) was 46% and 34%, respectively. Although results are promising considering from high-risk disease status of enrolled patients, relapse and infection are the main causes of death, suggesting that strategy for enhancing graft-versus-tumor effects is required to further improve the outcomes of stem cell transplantations after nonmyeloablative conditioning.

2.4 Donor Leukocyte Infusions for Residual Disease or Relapse

Recent work with patients who have relapsed or have persistent disease post allogeneic transplantation has centered on using DLIs in an effort to stimulate more GVT effect and re-attain remission. Reports are growing of clinically significant results in various types of malignant and non-malignant processes and it is now the standard of care to proceed with DLIs in patients who have relapsed disease post allogeneic transplantation.[28]

Barge and colleagues used a reduced intensity regimen with ATG for T cell depletion as well as alemtuzumab. All patients without severe toxicity received preemptive DLIs at a median of 6 months following transplant (11 of the 18 received at least 1 dose) and the authors contend this impacted long term engraftment and graft versus tumor effects.[29] The Atlanta group recently used preemptive DLI in all patients undergoing a T depleted reduced intensity transplant from a MUD donor. The study included 36 patients with median age of 59 years, 23 with varied myeloid malignancies, 13 lymphoid (CLL, Hodgkins, NHL) and 39% has low risk disease by the CIBMTR criterion. Twenty five of these patients had low donor % at day 60 and received the preemptive DLI. There were no cases of grade 3-4 aGVHD following DLI infusions, while chronic GVHD did occur in a number of patients (not separated by early or late DLI) and was severe in 12%. [30] The Boston group provided DLI following T cell depleted allografting preemptively 6-9 months following transplant noting apparent efficacy with improved PFS in those who were able to receive this cellular therapy.[31] Following T cell depleted allogeneic transplantation, a London team assessed the effect of preemptive DLI on outcomes for AML and MDS patients. Of the 62 who had a preemptive infusion, the majority had this within 6 months of the transplant. Despite this early infusion the aGVHD rate was only 31% and the 5 year survival was an encouraging 80%.[32]

For non-selected DLIs, donors are asked to provide peripheral blood progenitor cells in the same manner as for transplantation and these are then transfused through a peripheral intravenous line into the recipient. Typically, 10^5 to 10^6 CD3+ T cells/kg recipient weight are infused over 1 to 2 days, with 2-12 weeks between infusions, though there is no set standard. The lapse between infusions allows for observation and management of pancytopenia, the anticipated side effect if

significant GVT effects are encountered along with suppressive effects on the patient's innate hematopoiesis, before infusing the patient further. Immunosuppressive agents are kept stable during DLIs, however, if clinically significant GVHD does occur, treatment may be initiated to control the degree of response at that time.[33,34]

2.5 Immune Reconstitution and Anti-tumor Effects

The methods one chooses to monitor immune activity are difficult to correlate to clinical effects with the current state of knowledge. For this reason, our group uses multiple methods of measurement of immune system activity. Immune function of patients will be analyzed each pre- NK cell-enriched DLI, 1 day and 1 month post- NK cell enriched DLI, and at 3, 6 and 12 months during the first year after the last NK cell-enriched DLI to provide a sense of the pace and degree of recovery over time. This information is crucial to future plans of immune modulation following therapy. Analyses including WBC and differential to calculate absolute lymphocyte counts, enumeration of T, B, and NK subsets via multi-parameter flow cytometry will be performed as well as analysis on stool to determine the relationship between the gut microenvironment and GVHD [90]. Studies have shown that the microbiota may also modulate responses to immunotherapy, including CpG administration.[100] T cell blastogenic responses (3H-thymidine uptake) to plant mitogens [phytohemagglutinin (PHA), concavalin A (con A), pokeweed mitogen (PWM), or recall antigens (tetanus toxoid and *Candida Albicans*)] will be analyzed using Elispot and flow-cytometric cytokine assay. We will also perform Immunoscope and functional NK lysis assay.

2.6 Data with Non-Selected Donor Lymphocyte Infusions

Our data with non-selected DLIs shows the ability to safely infuse donor lymphocytes following non-myeloablative therapy. Sixty nine patients received at least one DLI dose of 1 x 10^4 - 3.27 x 10^8 CD3+ cells/kg at a median day of 50 days post transplantation for progressive or high-risk disease.[35] Our typical NK cell component of these infusions was 2-5 x 10^7 NK cells/kg patient weight. A median cell dose of 1 x 10^5 CD3+ cells/kg in the mismatched setting and 1 x 10^6 CD3+ cells/kg in the matched sibling setting appears safe with only 1 of 7 (14%) and 4 of 31 patients (13%), respectively, experiencing severe acute GVHD at these doses.

2.7 Rationale for Evaluating NK cell Infusion

NK cells are granular lymphocytes first identified in mice because of their capacity to rapidly lyse tumor cell targets. NK cells constitute approximately 5-15% of circulating lymphocytes and express CD16 and/or CD56. These cells lack the surface markers of either B or T cells, and were called "natural" killer cells by Kiessling et al because of their innate capacity for lysis. NK cells represent a generalized functional capacity for immune surveillance. Depletion of NK cells from animals enhances the growth in vivo of many implanted tumor cells.

NK cells are unique since they can mediate cytotoxicity without prior sensitization. As the majority of spontaneous tumors are typically nonimmunogenic, the elicitation of NK mediated MHC unrestricted cytotoxicity may be helpful in inducing significant antitumor responses. They are among the first immune cells to recover after stem cell transplantation, thus they may be the main GVT effectors in the early post-transplant period.[36,37,38,39,40] Selection of CD56+ NK cells using cell collection systems and CD56 monoclonal antibody has been demonstrated and reported.[41] While allowing for positive NK cell infusions in this manner, this process also accounts for a 3 to 4 log T cell depletion.[42] The ability to selectively collect NK cells for future immunotherapy may represent an innovative therapy in cancer treatment.

2.8 Our Data with This Procedure

We delivered 51 NK cell-enriched DLIs to 30 patients following a 3-6/6 HLA-matched T-cell-depleted nonmyeloablative allogeneic transplantation.[43] Eight weeks following transplantation, NK cell-enriched DLI were processed using a CD56+ selecting column with up to 3 fresh infusions allowed. Selection resulted in 96% (SD 8%) purity and 83% (SD 21%) yield in the matched setting and 97% (SD 3%) purity and 77% (SD 24%) yield in the mismatched setting (Table 1). The median number of CD3- CD56+ NK cells infused was 10.6 (SD 7.91) x 10^6 cells/kg and 9.21 (SD 5.56) x 10^6 cells/kg in the matched and mismatched setting, respectively. The median number of contaminating CD3+ CD56- T cells infused was 0.53 (SD 1.1) x 10^6 and 0.27 (SD 0.78) x 10^6 in the matched and mismatched setting, respectively. Only 1 patient each in the matched (n = 14) or mismatched (n = 16) setting experienced severe aGVHD with little other toxicity attributable to the infusions (Table 2 below). Long-term responders with multiple NK-cell-enriched DLIs and improved T cell phenotypic recovery had improved duration of responses (p = 0.0045) and overall survival (OS) (P = 0.0058). Our 1-step, high-yield process is feasible, and results in high doses of NK cells infused with little toxicity, and NK cell-enriched DLIs result in improved immune recovery and outcomes for some patients. Activation of NK cells in vivo after NK cell-enriched DLIs would be the next promising strategy to enhance immune recovery and anti-tumor activity and obtain better clinical outcomes.

TABLE 1 Cell dose infused post-processing

Donor	Cell Dose (Standard Deviation)	% Purity	% Yield	$CD3^+CD56^-$/kg × 10^5	$CD3^+CD56^+$/kg × 10^6	$CD3^-CD56^+$/kg × 10^6
Matched	Median (SD)	96 (8)	83 (21)	.53 (1.1)	1.94 (2.22)	**10.60 (7.91)**
Mis Matched	Median (SD)	97 (3)	77 (24)	.27 (.78)	3.67 (2.41)	**9.21 (5.56)**

Table 2

Toxicities for the matched sibling donors, N=14 (24 total NK cell enhanced infusions); Or mismatched family member donors, N=16 (27 total NK cell enhanced infusions)

(A) aGVHD								
Site↓/Grade→	HLA Matched N=14 patients; 24 total infusions				HLA Mismatched N=16; 27 total infusions			
	1	2	3	4	1	2	3	4
Skin	1	1	2	-	3	3	1	-
Gut	2	-	-	-	2	-	-	1
Liver		-	1	-		-	1	-
Overall	2	3	1	-	4	3	-	1

(B) Non- GVHD toxicity						
Organ System↓/CTC Grade→	Matched			Mismatched		
	2	3	4	2	3	4/5
Cardiac-arrythmia	1	-	-	2	-	-
Renal Insufficiency	-	-	-	1	-	-
Infectious	-	-	-		-	
Polyoma cystitis	1	1	-		2	-
CMV Reactivation only	3	-	-	6	-	-
CMV Disease	-	-	-	-	1	-
HSV	-	-	1	1	-	-
Parainfluenza	-	2	-	-	3	-
VZV	1	-	-	-	1	-
Bacterial	-	2	1	2	2	1
Parasitic (acanthamoeba)	-	-	1	-	-	-
Gastritis (non-GVHD)	2	-	-	-	-	-
Hemolytic Anemia		-	-	-	1	-
Pulmonary (non-infectious)	1	-	-	-		-
Post transplant lymphoproliferative disorder (PTLD)	-	-	-	-	-	2

1 case of severe cGVHD noted in the mismatched group

(Rizzieri DA, et al. Biol Blood Marrow Transplant. 2010 Aug;16(8):1107-14.)

2.9 Activation of NK cells directly by TLR ligand

TLRs belong to the family of pattern-recognition receptors that trigger innate immune responses providing both immediate protection against various pathogens and instructing the adaptive immune system by the induction of dendritic cell recruitment and maturation.[44,45] TLRs comprise 10 members so far identified in humans. Among these, TLR9 is highly expressed in plasmacytoid dendritic cells (pDCs) and B cells, and also expressed in NK cells.[46,47,48,49] The molecular structure recognized by TLR9 consists of unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotides in particular base contexts.[50] Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs bind to TLR9 and activate pDC and B cells.[51] TLR ligand can also directly activate NK cells. Hartmann et al. showed that ODNs containing specific CpG motifs up-regulated expression of the activation marker CD69 on NK cells and increased NK cell activity to lyse ^{51}Cr-labeled K562 cells.[52] An ODN with a TpC dinucleotides at the 5' end followed by three 6 mer CpG motifs (5'-GTCGTT-3') separated by TpT dinucleotides, that is ODN 2006 (CPG 7909), consistently showed the highest activity for human, chimpanzee, and rhesus monkey leukocytes. Sivori et al. demonstrated that in the presence of IL-12, freshly isolated NK cells responded to CPG 7909, and expressed CD69 and CD25 activation markers for all NK cell populations.[53] Further, NK cell stimulation also resulted in the induction of their functional activity as revealed by tumor necrosis factor-α and by up-regulation of cytolytic activity against tumor cells. As above, CpG ODNs, particularly CPG 7909, can directly activate NK cells through TLR9 signals.

2.10 Activation of NK cells by type I IFNs

Receptors of type I IFNs (IFNAR) are expressed in most tissues including NK cells.[54] In vitro experiment, type I IFNs enhance the ability of NK cells to kill target cells and to produce IFN-γ by indirect and direct mechanisms.[55,56] In mouse model, Gidlund et al. demonstrated that NK-cell-mediated cytotoxicity against sensitive target cell lines was elevated in mice treated with IFNs, and that activation of NK-cell-mediated lysis under these conditions was blocked by the administration of antibodies neutralizing IFN-α/β.[57] Using IFN receptor, IFNAR1- and IFNAR2-dificient mice, as well as IFNAR1-bloking antibody, Swann et al. demonstrated that endogenous type I IFN is critical for controlling NK cell-mediated antitumor response in many experimental tumor models.[58] The type I IFNs are also crucial for DC-induced NK-cell activation and have an important role in the induction of NK-cell cytotoxicity in vitro and in vivo.[59,60] TLR9-activated pDCs express high levels of costimulatory molecules, and secrete cytokines such as type I interferons, which then activate NK cells.

2.11 CpG infusion in vivo leads to secretion of type I IFNs

We recently showed that CpG infusion in vivo leads to secretion of type I IFNs in syngeneic transplantations model.[61] In this model, donor mice were transferred with HA-expressing A20 B-cell lymphoma (A20-HA) on day -25 and naive clone 4 HA-specific T cells on day -15. On day -10, recipients were injected with A20-HA. On day 0, splenocytes and bone stem cells were harvested from tumor-bearing donor mice. Recipient mice were irradiated and then injected with a graft composed of Lin-c-Kit+ stem cells and A20 lymphoma–purged splenocytes. Fourteen days after transplantation, mice were vaccinated with either TNF-α–matured DC-HA or DC-Con with (CpG in vivo) or without (no CpG) administration of CpG in vivo. Some mice received DCs matured with CpG ex vivo followed by HA-pulsing (CpG ex vivo). Sera were harvested 12 hours after administration of CpG and measured for IFN-α by ELISA. As shown in Figure 1C, the high levels of serum IFN-α was detected in the CpG in vivo group, while no

significant INF-α production was detected in no CpG or CpG ex vivo group (Figure 1C). This result demonstrated that CpG infusion in vivo is critical for secretion of type I IFNs in mice.

Figure 1

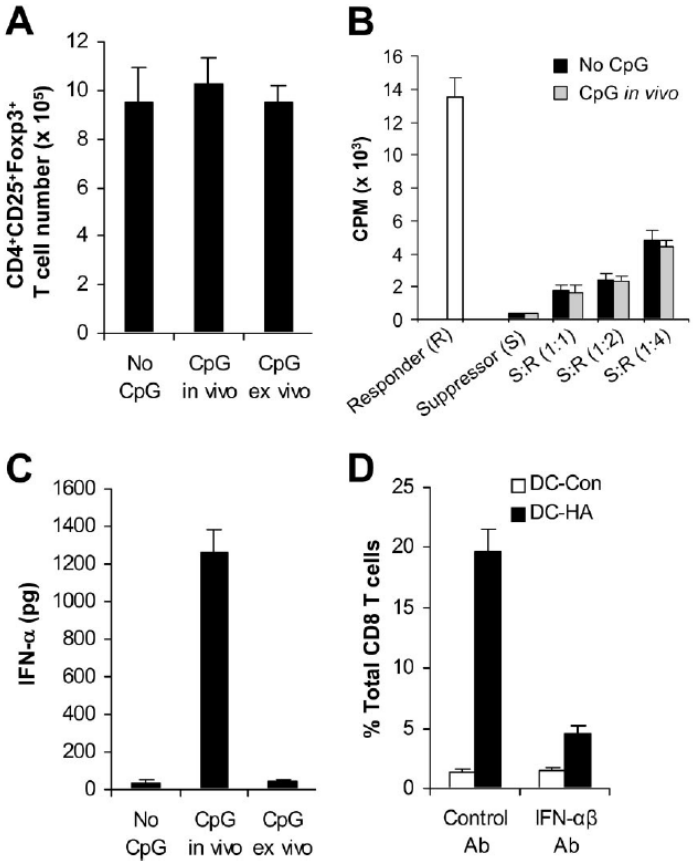

Figure 7. A critical role for type I IFN in CpG-dependent reversal of tumor-specific T tolerance after transplantation. (A-C) Syngeneic transplantations were set up as described in Figure 1A. Fourteen days after transplantation, mice were vaccinated with either TNF-α–matured DC-HA or DC-Con with (CpG in vivo) or without (no CpG) coadministration of CpG in vivo. Some mice received DCs matured with CpG ex vivo followed by HA pulsing (CpG ex vivo). Seven days after vaccination, the mean absolute number (± SD; n = 4) of $CD4^+CD25^+Foxp3^+$ T cells per spleen in each group is indicated (A). $CD4^+CD25^+$ T_{Reg} cells (Suppressor) were isolated by FACS sorting and assayed for their suppressive capacity on naive $CD4^+CD25^-$ T cells (Responder) at the indicated ratios of suppressor to responder (S:R) using an in vitro suppression assay. Cultures were labeled with [^{3}H]thymidine and harvested for scintillation counting. Results are expressed as mean CPM ± SD (B). Sera were harvested 12 hours after administration of CpG and measured for IFN-α by ELISA (C). (D) Fourteen days after transplantation, mice were vaccinated with DC-HA or DC-Con coadministered with CpG in vivo. Six hours before and 24 hours after vaccination, mice were treated with neutralizing antibodies to mouse IFN-α and IFN-β (IFN-αβ Ab) or a control Ab. Seven days after vaccination, splenocytes were stained with anti-CD8, anti-Thy1.1, and anti–IFN-γ antibodies, and the mean percentage (± SD; n = 4) of IFN-γ–secreting HA-specific T cells among total $CD8^+$ T cells is indicated (D). Representative results of 2 independent experiments are shown.

(Horkheimer I, et al. Blood. 2009 May 21;113(21):5330-9.)

2.12 CpG activates NK cells directly, leading to lysis of lymphoma cells in vitro

To assess the direct effect of CPG on NK cell cytotoxicity against tumor cells, we co-cultured luciferase-expressing A20 B-cell lymphoma cells with purified NK cells alone (control) or NK cells in presence of CpG, IL-18, or CpG + IL-18. A20-luciferase viability was determined by measuring luciferase activity. Compared to control, NK cell cytotoxicity was substantially enhanced by adding CpG to the media (Figure 2). This preliminary result suggests that CpG directly increases NK cell cytotoxicity, leading to lysis of lymphoma cells in vitro.

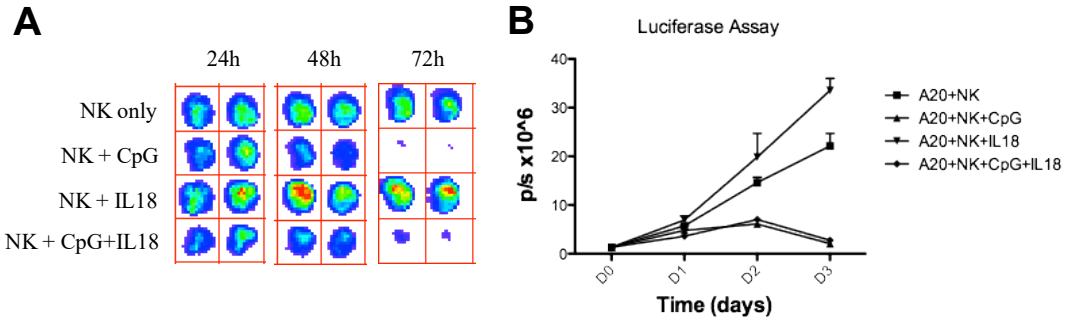

Figure 2. CpG directly activates NK cytotoxicity on tumor cells. Purified NK cells were co-cultured with Luciferase expressing A20 lymphoma cells (A20-luc) in presence of CpG (10 µg/ml), IL18 (20 ng/ml), or CpG + IL18. NK cells alone were used as a control. 3 days later, A20-luc viability was determined by imaging luciferase **(A)** and photons per second (p/s) were quantified and plotted **(B)**.

2.13 CPG 7909 is safely used in multiple clinical studies (agent identical to the one we will make for this study, DUK-CPG-001)

CPG 7909 is a synthetic, nuclease-resistant, TLR9-activating ODN that mimics unmethylated CpG motifs. Antitumor activity has been suggested after treatment with CPG7909 as a single agent or as part of combination therapy in patients with several tumor types, including metastatic malignant melanoma,[62,63,64] renal cell carcinoma,[65] non-small cell lung cancer,[66,67,68,69] and non-Hodgkin lymphoma.[70,71,72,73] Leonard et al. conducted Phase I trial of CPG 7909 with and following Rituximab in 50 patients with recurrent indolent and aggressive non-Hodgkin lymphoma.[74] Patients received Rituximab and CPG 7909 weekly for 4 weeks either intravenously (0.04, 0.16, 0.32, or 0.48 mg/kg) or subcutaneously (0.01, 0.04, 0.08, or 0.16 mg/kg). An additional extended-treatment cohort received 4 weeks of 0.24 mg/kg subcutaneously in combination with Rituximab followed by CPG 7909 alone subcutaneously weekly for 20 weeks. Most common adverse events were mild to moderate systemic flu-like symptoms and injection-site reactions. Objective responses occurred in 12 of 50 patients overall and in 6 of 12 patients in the extended treatment cohort, including 2 patients with Rituximab-refractory disease. Pashenkov et al.[75] conducted Phase II trial with a CPG7909 in 26 melanoma patients. CPG7909 was administered at a total dose of 6 mg subcutaneously once weekly in an outpatient setting for 24 weeks or until the development of progressive diseases. Laboratory and non-laboratory adverse events were limited, transient, and did not result in any withdrawals. Two patients experienced a confirmed partial response, and three patients experienced stable disease. Immunologic measurements revealed induction of an activated phenotype of pDC, elevation of a surrogate marker of serum type I IFN production, and significant stimulation of NK cell cytotoxicity, which was associated with clinical benefits. NK cytotoxicity showed divergent dynamics: an up to 30.1-fold increase was measured in 3 of 4 patients with partial response and stable disease and a decrease was observed in seven of the nine patients with progressive disease. This finding supported the findings in in vitro/in vivo study that CPG 7909 can activate NK cells directly through TLR9 or indirectly through type I IFNs signals.

From these findings, our identical agent DUK-CPG-001 with NK cell-enriched DLI could be a potentially effective strategy to enhance immune recovery and anti-tumor activity though NK cell activation and obtain better clinical outcomes.

2.14 Summary:

Graft-versus-tumor effects are critical in allogeneic stem cell transplantation, and can be enhanced by DLIs. However, the use of non-selected DLIs is limited by the risk of acute GVHD. We have shown that 1-step, high-yield process of NK cells is feasible, and results in high doses of NK cells infused with little toxicity. NK cell-enriched DLIs resulted in improved immune recovery and outcomes for some patients. Infusion of TLR9 agonist, CPG 7909, in mice leads to secretion of type I IFNs, which can activate NK cells. TLR ligand also directly activates NK cells. Our preliminary data showed that CPG 7909 directly activated NK cell function, leading to lysis of lymphoma cells. CPG 7909 has been already used in many studies, and shown to be safe. NK cells activation was also observed in clinical trials. In this study, we will evaluate the rates of progression-free survival and unacceptable toxicity in patients receiving NK cell-enriched DLIs when administered alone or administered with the TLR9 agonist, DUK-CPG-001, from a 8/8 HLA-matched related or unrelated donor (Cohort A) or 4-7/8 HLA-matched related donor (Cohort B) following allogeneic stem cell transplantation.

3 ELIGIBILITY CRITERIA

3.1 Inclusion criteria

1. Patients with hematologic diseases who have undergone an allogeneic transplantation, using a 7-8/8 HLA-matched related or unrelated donor or 4-6/8 HLA-matched related donor. This may include patients with a mixed chimeric state or disease persistence or at high risk of relapse (these are patients who do not fit exclusionary principle 3 below).

2. Performance status must be ECOG PS 0, 1, or 2.

3. Donor cellular engraftment of at least 2.5%.
4. < Grade 2 acute GVHD at time of the first NK cell-enriched DLI. Patients with treated acute GVHD must be on a stable dose of therapy (no increase in immunosuppressive therapy for the 2 weeks before planned NK cell-enriched DLIs). The dosage/level of immunosuppressive therapy at the time of NK-DLIs should be no greater than 20mg of prednisone daily or mycophenolate 500 mg bid daily or cyclosporine with a target level of 200 ng/mL or tacrolimus with a target level of 10 ng/ml.

5. Estimated survival of at least 8 weeks.

6. Age of >= 18 years.

7. Females of childbearing potential should have a negative serum beta-HCG test within 48 hours of beginning DLI and/or DUK-CPG-01 unless contraception is used after initial testing. *A female of childbearing potential (FCBP) is a sexually mature female who: 1) has not undergone a hysterectomy or bilateral oophorectomy; or 2) has not been naturally postmenopausal for at least 24 consecutive months (i.e., has had menses at any time in the preceding 24 consecutive months).*

8. Males must agree to use a medically acceptable form of birth control in order to be in this study and for 3 months after infusion

3.2 Exclusion criteria

1. Pregnant or lactating women.

2. Patients with other major medical or psychiatric illnesses, which the treating physician feels, could seriously compromise tolerance to this protocol.

3. Patients likely to have a significantly better durable response to allogeneic transplant alone (better than 60% progression free longer than 2 years) includes: those with myeloproliferative diseases or hemoglobinopathies with over 50% T cell subset engraftment (assessed around 100 days post transplant); It is not anticipated that any such patients would be transplanted within our program, however but those in first remission AML patients with good risk standard genetics or normal genetics with either NPM1 or CEBPA mutations, first chronic phase CML without kinase gene mutations, follicular lymphoma patients in first remission who only required 1 regimen to attain remission all would be excluded from this protocol.

3.3 HLA 4-8/8 matched related donor inclusion/exclusion criteria (criterion below are recommended but may evolve to follow current program standards) to be completed within 30 days of apheresis per standard guidelines

1. Adult donors must be an HLA 4-8/8 match with the patient
2. Potential donors under the age of 18 must have a single patient exemption approved by the IRB.
3. Donor must not have any medical condition which would make apheresis more than a minimal risk, and should have the following:
   (a) Family members will be considered for donation if they do not have a history of known cardiac problem and do not have abnormal cardiac findings by physical examination. Those with a history of cardiac problems or abnormal cardiac findings by physical examination should undergo a stress evaluation or be evaluated by a cardiologist and deemed eligible to donate.
   (b) Documented bilirubin and hepatic transaminases of $\leq$ 2.5 x ULN,
   (c) Documented adequate hematologic parameters including a hematocrit > 35% for males and 33% for females, white blood cell count of $\geq$ 3,000, and platelets ≥80,000.
   (d) FACT labs and final test results available prior to infusion into the patient (copy of labs included in appendices). In the second donation from the donor, the FACT labs must be redrawn within 30 days of initiation of apheresis. Positive serologies are not repeated as they remain positive for lifetime.

4. Females of childbearing potential should have a negative serum beta-HCG test within 48 hours of beginning apheresis unless contraception is used after initial testing. *A female of childbearing potential (FCBP) is a sexually mature female who: 1) has not undergone a hysterectomy or bilateral oophorectomy; or 2) has not been naturally postmenopausal for at least 24 consecutive months (i.e., has had menses at any time in the preceding 24 consecutive months).*

3.4 8/8 HLA matched unrelated donors will be matched at least as HLA -A, -B, C and -DRB1. Criterion for donation will be those allowing donation following the NMDP accepted donor criterion and program SOPs for the typical matched unrelated donors.

4. TREATMENT PLAN

4.1 Patient prior to starting therapy

Within 21 days prior to the 1st NK DLI, patients will be screened as follow:

- An updated medical history will be collected
- A physical examination performed including a pregnancy test, if the patient is a female of childbearing potential.
- Labs:
  - CBC (with differential preferred),
  - creatinine,
  - AST, ALT, and total bilirubin,
  - calcium,
  - LDH,
  - potassium,
  - sodium,
  - bicarbonate,
  - chloride and glucose,
  - albumin,
  - phosphorous,
  - magnesium and
  - alkaline phosphatase
  - Immune reconstitution panel
- Patients will have blood or marrow stored for chimerism analysis.
- Research blood samples for analysis of immune function.
- Stool will be collected for analysis of microbiota
- Tests documenting the patient's disease state:
- Bone marrow aspirate and biopsy for patients with known abnormalities of the marrow previous to the transplant should have tests repeated to check for the prior abnormalities such as morphology, flow cytometry, PCR, or cytogenetics.
  - Blood studies, and/or
  - Radiographic tests such as CTs, MRI, PET scans as determined by treating physician, for standardized response evaluations as outlined in section 4.3.2).

4.2 Donor apheresis and cell processing

Donors will return to the center for a fresh collection of NK cells and they will not need growth factor mobilization. One collection will be used for each NK cell infusion. Cells will be transfused immediately after collection and processing or the next day. Cell processing will be performed in our cryopreservation lab according to SOP for collection, labeling and handling. The cells will be NK selected using a CD56 antibody (CliniMACS CD56 Reagent), CliniMACS^{plus} instrument and CliniMACS tubing set provided by Miltenyi Biotec using the company protocol (Miltenyi Biotec Inc, Auburn, California). Pre and post processing cell count, viability, Hematopoietic Progenitor Cell Assay (HPCA) and flow analysis per SOP will be done.

***** Viability must be confirmed as ≥ 70% to release the product for infusion. Product with < 70% viability will not be infused into the patient. Repeat collection may be attempted at the treating physician's discretion. Similarly, CD56+ cell yield and purity will be available prior to infusion of cell product to make sure that the acceptance criteria are met. The acceptance criteria are ≥ 70% for purity and ≥ 40% for yield. The expected purity % and yield % is 96+/- 8 and 83 +/- 21 in 6/6 HLA-matched related donor, respectively, and 97+/- 3 and 77 +/- 24 in 3-5/6 HLA-

matched related donor, respectively.[76] Failure to meet the acceptance criteria for yield and purity will represent device performance failure which will be recorded, investigated and reported to the FDA.

4.3 Patient Evaluation

4.3.1 Patients must remain in clinic for at least 2 hours post-infusion for observation.

- Vital signs must be checked every 30 minutes

4.3.2 Assessment of disease will use standardized criteria and shall include a careful examination of the studies needed to detect the disease (for example, PET, radiographs, immuno-phenotype, marrow, molecular studies etc.).[77,78,79,80,81,82] Restaging may be altered at the discretion of the transplant physician following the patient (who are all subinvestigators in this study) if the patient is felt to be progressing before these time points but the recommended restaging is 3,6.and 12 months for 1 year after the last NK-enriched DLI, q6 months for the next 2 years, then as indicated clinically.

- ❖ Assessment of disease is required prior to each NK cell-enriched DLI, and 3,6, and 12 months for 1 year after the last NK-enriched DLI, then q6 month for the next 2 years.

- ❖ Immune reconstitution studies prior to and 1 day and 1 month after each NK cell-enriched DLI, and 3, 6, and 12 months after the last NK-enriched DLI.

- ❖ Determination of chimerism (by short tandem repeat analysis in use in our DUKE HLA laboratory with a 2% sensitivity) just prior to each NK cell-enriched DLI, and 3, 6, 12 months after the last NK-enriched DLI.

4.3.3 Toxicity will be formally evaluated by the protocol investigators, health care professionals directly under the supervision of protocol investigators, or physicians specifically trained in hematopoietic cell transplant will monitor the subjects' safety at appropriate intervals up until at least two years after NK infusion. Monitoring will occur 1 day post infusion and 1 month later (more as determined by treatment team) and, then 3, 6, and 12 months post-second DLI for the first year post-second DLI.

Assessments performed include the following for toxicity assessment following the NCI common toxicity criteria (version 4) and standard GVHD criteria (appendix I).:

- Medical history (specific to GI toxicity as well as overall new problems),
- Physical exam,
- CBC,
- Liver function tests (AST, ALT, bilirubin at a minimum), and
- Chemistry CS to include creatinine and BUN

The tests and procedures are not in addition or in excess of our standards for allogeneic transplants outside of this protocol. The transplant team is available for constant communication with the patients and their local physicians to monitor their toxicities. Further follow up at the transplant center will be required as needed if the patient has a toxicity due the transplant or infusion procedures. Patients with a grade 3 or greater toxicity

due to the study will be seen every other week at a minimum until the toxicity is < grade 3, and then will be seen as clinically appropriate.

NOTE: If a patient does not receive the 2nd DLI, they will be followed every 3 months after their 1st DLI.

4.3.4 Stool Collection and Microbiome Analysis:

We will collect stool samples from patients at the following time points throughout the study: pre-DLI, 1 month post, pre-second DLI, 1 month post, then at 3, 6 and 12 months after last DLI for one year. Stool samples from patients may be stored at 4°C for up to 24 h before freezing at -80°C for batch analysis. Samples not used during this research will be stored in the Duke Data and Specimen Repository (IRB Pro00006268) unless the patient does not consent to the Repository. If no consent to store the samples is obtained, the samples will be destroyed after study analyses are complete.

To analyze the gut microbiome, DNA will be extracted from fecal samples[91] and gut flora bacterial density will be quantified using real-time quantitative polymerase chain reaction (qPCR) as described.[92] We will amplify 16S ribosomal RNA (rRNA) using Illumina HiSeq platform and analyze the data using the Qiime script package with parallel processing.[93] Sequences will be de-noised and clustered at 97% identity using USEARCH and aligned to the 16S rRNA gene, using the align.seqs.py wrapper with the PyNAST algorithm and Greengenes reference alignment. Based on these results, we will calculate diversity (Shannon Diversity, primary endpoint, and Chao1) and construct phylogenetic trees using computational analysis software.

4.4 Patient NK cell infusion and CpG administration plan

This treatment regimen may be administered on an outpatient basis.

4.41 NK cell-enriched DLI:

The target cell dose for NK cell-enriched DLI will be as many cells as can be collected with less than 0.5 x 10^6 CD3+ CD56- cells/kg patient weight in the 4-6/8 HLA-matched related setting and 1 x 10^6 CD3+ CD56- cells/kg patient weight in the 7-8/8 HLA-matched related or unrelated setting. The first NK cell-enriched DLI will be administered between one to six months post-transplant. The second NK cell-enriched DLI will be administered one to three months post the first infusion, in patients who have < grade II aGVHD at the time of infusion and have not had unacceptable toxicities (see 6.2.1 Definition of unacceptable toxicity) that are at least possibly related to the previous DLI and resolved to grade 1 or less. The second DLI will NOT be administered in patient with ≥grade III aGVHD at the time of infusion or unacceptable toxicities at least possibly related to the first infusion. If patients have GVHD and were on immunosuppressive therapy at study entry, patients will continue on their stable dose of immunosuppressive agents started for therapy of acute GVHD before the NK cell-enriched DLI, and will not taper until at least 6 weeks following the each NK cell-enriched DLI (unless disease progression or patient toxicity from the agents requires earlier taper). The donor NK cells will be infused over 30 minutes. Diphenhydramine 25 mg iv or po, and Acetaminophen 650 mg po will be used prior to each reinfusion, unless there is a history of allergy or contraindication in the patient, in which case hydrocortisone 50mg IV will be used.

These cells are infused into the patient via a peripheral intravenous line or central line. If signs of GVHD occur after NK cell-enriched DLI, immunosuppressive agents (prednisone, cyclosporine, tacrolimus and/ or mycophenolate preferred first choices) may be started.

4.42 Adverse effect of NK cell-enriched DLI:

We previously delivered 51 NK cell-enriched DLIs to 30 patients following a 3-6/6 HLA-matched T-cell-depleted nonmyeloablative allogeneic transplantation.[83] In this study, mild skin aGVHD was experienced by 2 of the 14 matched patients prior to treatment on this study (Table 2A). Six of these patients experienced equal to or greater than grade 1 aGVHD, although only 1 had grade 3-4 (severe) overall aGVHD. The median onset following the first NK cell infusion was 2 months (range: 1-7 months). Mild skin aGVHD was experienced by 4 of the 16 mismatched patients prior to enrollment in this study. At the doses provided, equal to or greater than grade 1 aGVHD was experienced by 8 of the 16 patients, with skin GVHD being common, although only 1 of the 16 had grade 3-4 (severe) overall aGVHD. The median onset of aGVHD following the first NK cell infusion was 1.5 months (range: 1-5 months). Only 1 case of severe chronic GVHD (cGVHD) was encountered. Severe non-aGVHD toxicity was uncommon (Table 2B). Only 1 subject in each group (matched and mismatched) had bacterial sepsis, although viral exanthemas remained a significant concern despite the lymphocyte infusions with Polyoma, cytomegalovirus (CMV), varicella zoster virus (VZV), herpes simples virus (HSV), and Parainfluenza all encountered. Three cases of cardiac dysrhythmias (atrial) needing medication for rate control were documented and 1 case of transient renal insufficiency. There was 1 case of significant decrease in donor engraftment, possibly leading to secondary graft failure, not because of evident disease progression.

Table 3:

Table 2. Toxicities for the Matched Sibling Donors, N = 14 (24 Total NK Cell-Enhanced Infusions); or Mismatched Family Member Donors, N = 16 (27 Total NK Cell-Enhanced Infusions)

(A) aGVHD

	HLA Matched N = 14 Patients; 24 Total Infusions				HLA Mismatched N = 16; 27 Total Infusions			
Site↓ / Grade→	1	2	3	4	1	2	3	4
Skin	1	1	2	—	3	3	1	—
Gut	2	—	—	—	2	—	—	1
Liver		—	1	—		—	1	—
Overall	2	3	1	—	4	3	—	1

(B) Non-GVHD toxicity

	Matched			Mismatched		
Organ System↓ / CTC Grade→	2	3	4	2	3	4/5
Cardiac-arrythmia	1	—	—	2	—	—
Renal Insufficiency	—	—	—	1	—	—
Infectious	—	—	—		—	
Polyoma cystitis	1	1	—		2	—
CMV reactivation only	3	—	—	6	—	—
CMV disease	—	—	—	—	1	—
HSV	—	—	1	1	—	—
Parainfluenza	—	2	—	—	3	—
VZV	1	—	—	—	1	—
Bacterial	—	2	1	2	2	1
Parasitic (acanthamoeba)	—	—	1	—	—	—
Gastritis (non-GVHD)	2	—	—	—	—	—
Hemolytic anemia		—	—	—	1	—
Pulmonary (noninfectious)	1	—	—	—		—
Posttransplant lymphoproliferative disorder (PTLD)	—	—	—	—	—	2

GVHD indicates graft-versus-host disease; VZV, varicella zoster virus; HSV, herpes simplex virus; CMV, cytomegalovirus; NK, natural killer.
One case of severe cGVHD noted in the mismatched group.

(Rizzieri DA, et al. Biol Blood Marrow Transplant. 2010 Aug;16(8):1107-14.)

4.5 DUK-CPG-001

Patients are randomized into two arms: patients who receive NK cell-enriched DLI alone and patients who receive NK cell-enriched DLI with DUK-CPG-001, an agent chemically identical to CPG 7909 and this data is what is presented below for potential toxicities.

4.51 DUK-CPG-001 preparation

DUK-CPG-001 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') was synthesized with a wholly phosphorothioate backbone in GMP facility of Agilent Technologies (Boulder, CO). Agilent Technologies produced DUK-CPG-001 that complies with the pharmaceutical GMP guidelines for use as active pharmaceutical ingredients (API) in clinical trials.

Bulk DUK-CPG-001 is resuspended in normal saline with a final concentration of 10 mg/mL, aliquoted into 0.75 mL (7.5 mg) tubes and stored at -20°C in the Investigational pharmacy at Duke.

On the day of NK cell-enriched DLI, for those patients who are randomized to receive DUK-CPG-001, 0.75 mL (7.5 mg) aliquots of DUK-CPG-001 will be dispensed to nurse, upon request, by the GMP facility personnel. DUK-CPG-001 will be thawed at room temperature right before use and a 5.0 mg dose will be injected intravenously right after

NK cell-enriched DLI. DUK-CPG-001 should be given as an IV push within 5 minutes of administration.

DNA oligonucleotides are very stable. A similar approved drug, Kynamro, is a DNA oligo and it can be left 30 degrees C for 14 days. Thus our plan is to use DUK-CPG-001 within 4 hours of thawing.

4.52 DUK-CPG-001 storage and accountability

Storage:
DUK-CPG-001 is stored at -20ºC freezer until use in the Investigational Chemotherapy Services pharmacy at Duke.

Accountability:
The ICS is responsible for establishing study‐specific procedures for appropriate drug accountability, billing, procurement, storage, preparation, dispensing and destruction of all investigational drugs within the Cancer Center. These procedures comply with local, state and federal requirements and are consistent with practice standards of the American Society of Health‐System Pharmacists and the Joint Commission on Accreditation of Healthcare Organizations. Investigational drugs are accounted for using the National Cancer Institute's Investigational Drug Accountability Record (DAR). All drug transfers, receipts and disposal are recorded on the DAR. Prepared, but unused, IV medications will be destroyed in accordance with requirements of the principal investigator, the sponsor and applicable OSHA and EPA regulations. Documentation of destruction is made on the DAR.

4.53 DUK-CPG-001 transport and administration

Transport:
Study medications that are to be transported to another pharmacy in the hospital are transported by either a member of the Investigational Chemotherapy Service or by the Stem Cell Transplant Courier. If the medication is refrigerated or frozen, drug is transported with ice and immediately delivered to the appropriate satellite.

Administration:
Using standard institutional guidelines, 5.0 mg of DUK-CPG-001 will be injected intravenously right after the NK cell-enriched DLI. DUK-CPG-001 is expected to be stable within 4 hours of thaw.

4.54 Adverse effects of CPG7909, which is identical to DUK-CPG-001

The primary adverse events of CPG7909, which is identical to DUK-CPG-001, are dose-dependent local injection reactions (such as erythema, pain, swelling, induration, pruritus or warmth at the site of injection) when subcutaneously administered or systematic flu like reactions (such as headache, rigors, myalgia, pyrexia, nausea and vomiting) (Table 3).[84] The most commonly reported treatment-related hematologic toxicities are lymphopenia, leukopenia, neutropenia, anemia, and thrombocytopenia. Dyspnea, syncope, and hypotension are also reported.[85] Published >=Grade 3 adverse effects of CPG 7909 combined with or without any chemotherapy/radiotherapy are shown in Table 3.

Table 3A: Grade3/4 Adverse effect of CPG7909 (monotherapy)	Pashenkov, et al (Ref 51)	Weber, et al (Ref 53)	Thompson, et al (Ref 54)	Yamada, et al (Ref 56)	Kim, et al (Ref 59)	Link, et al (Ref 62)
	n=20	n=92	n=39	n=12	n=28	n=23
General disorders and administraion site conditions						
Injection-site reactions			1 (3%)			1 (4%)
Fatigure			4 (10%)		4 (14%)	
Malaise			1 (3%)			
Pyrexia			1 (3%)			
Rigors			1 (3%)			
Asthenia		2 (2%)				
General physical health deteriorartion		2 (2%)				
Metabolism and Nutrition Disorder						
Anorexia			1 (3%)			
Hypophosphatemia			1 (3%)			
Dehydration		1 (1%)				
Gastrointestinal disorders						
Nausea		2 (2%)				
Vomiting		1 (1%)				
Nervous system disorders						
Dizziness					1 (4%)	
Memory impairment			1 (3%)			
Amnesia		1 (1%)				
Psychiatric disorders						
Confusion			1 (3%)			
Skin and subcutaneous tissue disorders						
Night sweats		1 (1%)				
Urticaria		1 (1%)				
Hematologic and lymphatic disorders						
Anemia		4 (4%)				3 (13%)
Leukopenia					1 (4%)	
Lymphopenia		5 (5%)		1 (8%)	4 (14%)	3 (13%)
Neutropenia	1 (5%)	2 (2%)			2 (7%)	2 (9%)
Febrile neutropenia		1 (1%)				
Thrombocytopenia						4 (17%)
Laboratory value abnormalities						
Increased LDH	1 (5%)					
Increased ALT						1 (4%)
Increased γ-GTP					1 (4%)	
Neoplasms benign, malignant and unspecified						
Chronc lymphatic leukemia	1 (5%)					
Ear and labyrinth disorders						
Deafness neurosensory	1 (5%)					
Musculoskeletal disorders						
Peripheral swelling	1 (5%)					
Back pain					2 (7%)	
Arthralgia			1 (3%)			
Muscle spasms			1 (3%)			
Myalgia			1 (3%)			
Polyarthralgia			1 (3%)			
Pain in extremity		2 (2%)				
Infections and infestations						
Pneumonia		1 (1%)				
Postprocedural site wound infection	1 (5%)					
Respiratory, thracic, and mediastinal disorders						
Dyspnea		4 (4%)			1 (4%)	2 (9%)
Pleural effusion		2 (2%)				
Vascular Disorder						
Hypotention		1 (1%)	1 (3%)			
Renal and urinary disorders						
Potassium	1 (5%)					
Sodium	1 (5%)					
Uric acid	1 (5%)					
Coagulation						
PT	1 (5%)					
PTT	1 (5%)					
Immune system						
Anaphylaxis		2 (2%)				

Table 3B: Grade3/4 Adverse effect of CPG7909 (combination therapy including cytotoxic chemotherapy)	Weber, et al (Ref 53)	Manegold, et al (Ref 55)	Yamada, et al (Ref 56)	Manegold, et al (Ref 57)	Hirsh, et al (Ref 58)	Brody, et al (Ref 60)	Leonard, et al (Ref 61)
	W/cytotoxic chemo Tx	W/cytotoxic chemo Tx	W/cytotoxic chemo Tx	W/cytotoxic chemo Tx	W/cytotoxic chemo Tx	W/RadioTx	W/Rituximab
	n=45	n=75	n=12	n=393	n=384	n=15	n=31
General disorders and administraion site conditions							
Injection-site reactions		5 (7%)	1 (8%)	41 (10%)	18 (5%)		
Fatigure		5 (7%)		31 (8%)	27 (7%)		
Pyrexia				7 (2%)	5 (1%)		
Weakness		3 (4%)					
Asthenia				16 (4%)	10 (3%)		
Flu-like symptomes			2 (17%)	18 (5%)	12 (3%)		
Constipation				2 (1%)	2 (1%)		
Peripheral edema				2 (1%)			
Metabolism and Nutrition Disorder							
Anorexia			2 (7%)	14 (4%)	8 (2%)		
Hypocalcemia							1 (3%)
Hypokalemia							1 (3%)
Dehydration		3 (4%)					
Gastrointestinal disorders							
Nausea	2 (4%)	2 (3%)		17 (4%)	13 (3%)		
Diarrhea		5 (7%)		10 (3%)	7 (2%)		
Vomiting	2 (4%)	1 (1%)		25 (6%)	8 (2%)		
Abdominal pain		2 (3%)					
Gastroesophageal reflux	1 (2%)						
Nervous system disorders							
Peripheral neuropathy		2 (3%)			23 (6%)		
Convulsions							1 (3%)
Parathesia					2 (1%)		
Headache				4 (1%)	2 (1%)		
Psychiatric disorders							
Depression							1 (3%)
Skin and subcutaneous tissue disorders							
Urticaria	1 (2%)						
Alopecia					12 (3%)		
Erythema					2 (1%)		
Rash				3 (1%)			
Hematologic and lymphatic disorders							
Anemia	3 (7%)	11 (15%)	3 (25%)	150 (38%)	57 (15%)		1 (3%)
Leukopenia	5 (11%)	36 (48%)	7 (58%)	208 (53%)	159 (41%)		
Lymphopenia	9 (2%)	18 (24%)	4 (33%)	87 (22%)	92 (24%)		1 (3%)
Neutropenia	4 (9%)	54 (72%)	11 (91%)	291 (74%)	281 (73%)		2 (6%)
Febrile neutropenia	2 (4%)	3 (4%)					
Infections		1 (1%)					
Thrombocytopenia	2 (4%)	14 (19%)	2 (17%)	267 (68%)	66 (17%)		
Hemoptysis		2 (3%)					
Blood production transfusion							1 (3%)
Laboratory value abnormalities							
Hepatic funciton abnormal							1 (3%)
Hyperbilirubinemia							1 (3%)
Neoplasms benign, malignant and unspecified							
Malignant neoplasm progression							1 (3%)
Musculoskeletal disorders							
Back pain					11 (3%)		
Arthralgia					9 (2%)		
Myalgia					2 (1%)		
Pain in extremity				8 (2%)	8 (2%)		1 (3%)
Infections and infestations							
Bacteremia							1 (3%)
Cardian disorders							
Cardiac failure							1 (3%)
Chest pain					8 (2%)		
Respiratory, thracic, and mediastinal disorders							
Dyspnea	2 (4%)			30 (8%)			1 (3%)
Pleural effusion	1 (2%)						
Cough				5 (1%)	5 (1%)		
Vascular Disorder							
Hypertention							1 (3%)
Deep venous thrombosis							2 (6%)
Renal and urinary disorders							
Ureteric obstruction							1 (3%)
Urinary retention							1 (3%)
Acyte renal filure							1 (3%)
Coagulation							
PTT							1 (3%)

4.6 Dose delays and modifications

4.61 Delayed NK cell-enriched DLI and DUK-CPG-001 administration

Due to clinical concerns for the patients such as concern over GVHD, infection, or other clinical reasons, the NK cell-enriched DLI with or without DUK-CPG-001 may be altered by up to $\pm$ 4 weeks. However, the NK cell-enriched DLI will not be administered earlier than 4 weeks post transplant.

5.0 PATIENT REGISTRATION

All patients must be registered with the study coordinator and data coordinator. The eligibility checklist must be complete and the IRB approved consent form signed before any protocol therapy may be started on the patient or donor.

6.0 STUDY DESIGN AND ANALYSIS

This is a randomized, parallel phase II study of NK cell-enriched DLIs when administered alone or administered with the TLR9 agonist, DUK-CPG-001, from a 7-8/8 HLA-matched related/unrelated donor (Cohort A) or 4-6/8 HLA-matched related donor (Cohort B) following allogeneic stem cell transplantation.

In Cohort A, a total of 50 patients will be randomized with equal allocation to two arms defined by whether or not a TLR9 ligand, DUK-CPG-001, is administered with NK cell-enriched DLIs. In Cohort B, a total of 50 patients will be randomized with equal allocation to the two arms. Stratification will be done within disease types (myeloid or lymphoid diseases) in each cohort. A block randomized design is used to randomize patients to the two treatments within each stratum for each cohort. Primary endpoints are analyzed separately in Cohort A and B.

6.1 Primary Objectives 1 and 2

1. To evaluate the one-year progression-free survival rates in patients receiving NK cell-enriched DLI administered alone from a 4-6/8 HLA-matched related or 7-8/8 HLA-matched donor following allogeneic stem cell transplantation.

2. To evaluate the one-year progression-free survival rates in patients receiving NK cell-enriched DLI administered with a TLR9 ligand, DUK-CPG-001, from a 4-6/8 HLA-matched related or 7-8/8 HLA-matched donor following allogeneic stem cell transplantation

6.1.1 Definition of progression-free survival

Progression-free survival is defined from the day of the first NK cell-enriched DLI until the day of disease relapse, disease progression, or death whichever comes first. Patient without relapse, progression, or death will be censored at the last follow-up.

6.1.2 Statistical considerations

Cohort A (7-8/8 HLA-matched related or unrelated transplantation)

A 2-stage MiniMax design[86] will be used to test the null hypothesis that the progression-free survival rate is $\leq 46\%$ against the alternative hypothesis that it is $\geq 71\%$ in each arm. Nine patients will be treated in the first stage. If the number of patients who are alive without relapse/progression is equal to 4 or less at 1 year after transplantation in one arm, then the study for the arm will be terminated and the treatment is ineffective. If there are at least 5 patients (56%) who are alive without relapse/progression at 1 year after transplantation, the sample size will be increased up to a maximum of 25 patients. If the number of patients who are alive without relapse/progression is

equal to 15 or less at 1 year after transplantation, then the treatment is ineffective. If there are at least 16 patients (64%) who are alive without relapse/progression at 1 year after transplantation, this protocol will be deemed effective. This design has a significance level of 0.05, a power of 0.80, a probability of early termination under the null of 0.60, and an expected sample size under the null of 16 patients.

Table 4

Stage	Total Accrual	Close study/therapy if number of patients who are alive without relapse/progression is ≤
1	9	4 (44%)
2	25	15 (60%)

Cohort B (4-6/8 HLA-matched related transplantation)
A 2-stage MiniMax design will be used to test the null hypothesis that the progression-free survival rate is ≤ 34% against the alternative hypothesis that it is ≥ 59% in each arm. Twelve patients will be treated in the first stage. If the number of patients who are alive without relapse/progression is equal to 4 or less at 1 year after transplantation in one arm, then the study for the arm will be terminated and the treatment is ineffective. If there are at least 5 patients (42%) who are alive without relapse/progression at 1 year after transplantation, the sample size will be increased up to a maximum of 25 patients. If the number of patients who are alive without relapse/progression is equal to 12 or less at 1 year after transplantation, then the treatment is ineffective. If there are at least 13 patients (52%) who are alive without relapse/progression at 1 year after transplantation, this protocol will be deemed effective. This design has a significance level of 0.05, a power of 0.80, a probability of early termination under the null of 0.61, and an expected sample size under the null of 17 patients.

Table 5

Stage	Total Accrual	Close study/therapy if number of patients who are alive without relapse/progression is ≤
1	12	4 (33%)
2	25	12 (48%)

6.2 Primary Objective 3 and 4

3. To evaluate the rates of unacceptable toxicity of using NK cell-enriched DLI administered alone from a 7-8/8 HLA-matched related or unrelated donor or 4-6/8 HLA-matched related donor following allogeneic stem cell transplantation.

4. To evaluate the rates of unacceptable toxicity of using NK cell-enriched DLI administered with TLR9 ligand, DUK-CPG-001, from a 7-8/8 HLA-matched related or unrelated donor or 4-6/8 HLA-matched related donor following allogeneic stem cell transplantation.

6.2.1 Definition of unacceptable toxicity

GVHD will be graded as standard criteria and other toxicity will be graded as per NCI common toxicity criteria (v.4) (appendix I). Unacceptable toxicity is defined as any of the following related to the DLI procedure:

(a) Grade ≥ III aGVHD of the gut or liver or Grade IV aGVHD of the skin lasting > 7 days;
(b) Grade 4 toxicity from the procedure in the cardiac, dermatologic, gastrointestinal, hepatic, pulmonary, renal/genitourinary, or neurologic categories that lasts > 5 days;
(c) Treatment-related death caused by the toxicities related to DLI procedure.

6.2.2 Statistical considerations

Rate of unacceptable toxicity will be calculated and monitored with the stopping rules based on 2-stage MiniMax design in the table below. For this early stopping rule, we will only include the rate of unacceptable toxicities from the first infusion. Some patients will receive the second planned DLI. If the rate of unacceptable toxicity for the second DLI exceeds these same boundaries as the first infusion, then the protocol will be amended to delete the plan for the second DLI. Toxicity in the other categories will be informally monitored and tabulated by Type and Grade, but they will not be used to determine tolerability of the procedure.

For purposes of toxicity monitoring, each patient will be followed for an unacceptable toxicity for 8 weeks after each NK cell-enriched DLI. Accrual will not be suspended while collecting data for the interim analyses. However, as soon as a boundary within a stage in the table above is crossed, the study will be closed to further accrual.

Patients will be stratified according to T cell depletion usage or not. Analysis within strata is not planned.

Cohort A (7-8/8 HLA-matched related or unrelated transplantation)

A 2-stage MiniMax design will be used to test the null hypothesis that the unacceptable toxicity rate is $\geq$0.40 against the alternative hypothesis that it is $\leq$ 0.15 in each arm. Seven patients will be treated in the first stage. If the number of patients with an unacceptable toxicity is equal to 3 or more at 16 weeks after the first NK cell enriched-DLI in one arm, then the study for the arm will be terminated. If there are at most 2 patients (29%) with an unacceptable toxicity at 16 weeks after the first NK cell enriched-DLI, the sample size will be increased up to a maximum of 20 patients. If the number of patients who are alive without relapse/progression is equal to 5 or more at 16 weeks after the first NK cell enriched-DLI, then the study for the arm will be terminated. If there are at most 4 patients (20%) with an unacceptable toxicity at 16 weeks after the first NK cell enriched-DLI, this protocol will be deemed safe and continue to accrue to a total of 25 patients. This design has a significance level of 0.05, a power of 0.80, a probability of early termination under the null of 0.58, and an expected sample size under the null of 12 patients.

Table 6

Stage	Total Accrual	Close study/therapy if number of patients with unacceptable toxicity is $\geq$
1	7	3 (43%)
2	20	5 (25%)

Cohort B (4-6/8 HLA-matched related transplantation)

A 2-stage MiniMax design will be used to test the null hypothesis that the unacceptable toxicity rate is $\geq$0.45 against the alternative hypothesis that it is $\leq$ 0.20 in each arm. Nine patients will be treated in the first stage. If the number of patients with an unacceptable toxicity is equal to 4 or more at 16 weeks after the first NK cell enriched-DLI in one arm, then the study for the arm will be terminated. If there are at most 3 patients (33%) with an unacceptable toxicity at 16 weeks after the first NK cell enriched-DLI, the sample size will be increased up to a maximum of 23 patients. If the number of patients who are alive without relapse/progression is equal to 7 or more at 16 weeks after the first NK cell enriched-DLI, then the study for the arm will be terminated. If there are at most 6 patients (26%) with an unacceptable toxicity at 16 weeks after the first NK cell enriched-DLI, this protocol will be deemed safe and continue to accrue to a total of 25 patients. This design has a significance level of 0.05, a power of 0.80, a probability of early termination under the null of 0.64, and an expected sample size under the null of 14 patients.

Table 7

Stage	Total Accrual	Close study/therapy if number of patients with unacceptable toxicity is ≥
1	9	4 (44%)
2	23	7 (30%)

6.3 Secondary Objectives

1. To evaluate the recovery of immune cell populations pre and post infusion in patients receiving NK cell-enriched DLI administered alone or administered with TLR9 ligand, DUK-CPG-001.

2. To evaluate the immune function with antigen specific recovery with Elispot, Immunoscope, and flow-cytometric cytokine assay, and functional NK lysis assay, pre and post infusion in patients receiving NK cell-enriched DLI administered alone or administered with TLR9 ligand, DUK-CPG-001.

The repeated measurements of immune cell numbers and immune function will be plotted against time for each patient. The means of the measurements across patients will calculated with their 95% confidence intervals. These means will also be plotted against time to show the change with time. The generalized estimating equation method will be used to estimate the rates of change of the mean cell numbers and mean immune function over time, along with their 95% confidence intervals.

6.31 Secondary objectives 1 and 2:
Evaluation of the recovery of immune cell populations pre and post infusion will be performed.

Immunephenotype
NK cell: CD3-CD56^{bright}CD16-, CD3-CD56^{bright}CD16+, CD3-CD56^{dim}CD16+
CD3+ T cell: CD3+
CD4+ T cell: CD3+CD4+
CD8+ T cell: CD3+CD8+
γδ T cells: CD3+ γδTCR+
B cell: CD19+, CD3-, CD16-, CD56-
Regulatory T cell: CD4+, CD25+, CD62L+
Cytotoxic T cell: CD8+, CD57+, CD28-
Activated T cell: CD8+, HLA-DR+
Recent thymic emigrants: CD4+, CD45RA+/CD45RO-, CD62L+
Plasmacytoid dendritic cell: CD123+, CD11c-
Myeloid dendritic cell: CD123-, CD11c+
NKT cell: CD3+, CD16+/CD56+

Quantification of these lymphoid subsets were performed by flow cytometry on fresh peripheral blood prior to, and then at 1 day, and ,1 month after each NK cell enriched DLI and at 3, 6, and 12 months followingthe last NK enriched DLI.[87,88] The median number of these cell populations will be tabulated at each time points. A plot of number of these against time point will be drawn, using a separate line for each patient.

Evaluation of the immune function with antigen specific recovery with Elispot, Immunoscope, and flow-cytometric cytokine assay, and functional NK lysis assay, pre and post infusion will be performed prior to, and then at 1 day, and 1 month after each NK cell enriched DLI ,and at 3, 6, and 12 months followingthe last NK enriched DLI. The median number will be tabulated at each time points. A plot of number of these against time point will be drawn, using a separate line for each patient.

Analysis of the Microbiota**:** Shannon diversity index will be compared using unpaired two-sided Student's *t* tests with a more stringent cut-off of 0.0125 given multiple comparisons, by the Bonferroni correction for 4 time periods of independent comparisons. Comparisons of bacterial populations will be made by using the Adonis non-parametric method from within the Qiime package using UnifFrac distance matricies from the populations being compared. P values will be calculated with $\alpha = 0.05$. Metatstats will be used for further statistical analyses of population structure, membership, and diversity with metadata such as time from transplant and dietary measurements such as caloric intake [94]. Changes in specific bacterial families of interest will be compared using a two-sided Student's *t* test, with normality confirmed by D'Agostino and Pearson omnibus test with $p \leq 0.05$. All other comparisons will be done using two-sided Mann-Whitney tests. The R packages vegan[95], phyloseq [96], APE [97], randomForest [98], and arules [99] will be used for identification of associative patterns between taxa most associated with GVHD and disease responses.

6.3.1.1 Evaluation of NK cell function

NK cell function will be measured by flow-based assay as described in our previous study.[89] Briefly, K562 and Raji cells are exposed to varying ratios of CD56+ NK cells enriched form the patient's peripheral blood. In each assay, the total number of target cells is held constant and 3-fold serial dilutions of the effector cells, performed in triplicate, are established. The percent lysis is measured on the target cells directly as a percentage of 7AAD+ cells. Background (minimum) 7AAD uptake is calculated from targets incubated without effectors. The percent lysis (NK cell lytic activity) is simply calculated as: (% sample 7AAD uptake - %minimum 7AAD uptake).

The NK functional response 1 day and 1 month post NK cell-enriched DLI is defined as percent increase of NK cell lytic activity at 1 day and 1 month post NK cell-enriched DLI compared with NK cell lytic activity pre NK cell-enriched DLI.

7.0 SAFETY MONITORING AND REPORTING

The PI is responsible for the identification and documentation of adverse events and serious adverse events, as defined below. At each study visit, the PI or designee must assess, through non-suggestive inquiries of the subject or evaluation of study assessments, whether an AE or SAE has occurred.

7.1 Adverse Events

An adverse event (AE) is any untoward medical occurrence in a subject receiving study drug and which does not necessarily have a causal relationship with this treatment. For this protocol, the definition of AE also includes worsening of any pre-existing medical condition. An AE can therefore be any unfavorable and unintended or worsening sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of study drug, whether or not related to use of the study drug. Abnormal laboratory findings without clinical significance (based on the PI's judgment) should not be recorded as AEs. But laboratory value changes that require therapy or adjustment in prior therapy are considered adverse events.

From the time the subject is screened through the End of Study visit (as defined in Section 10.6), all AEs must be recorded in the subject medical record and adverse events case report form.

AEs will be assessed according to the CTCAE version 4.0. If CTCAE grading does not exist for an AE, the severity of the AE will be graded as mild (1), moderate (2), severe (3), life-threatening (4), or fatal (5).

Attribution of AEs will be indicated as follows:

- Definite: The AE is clearly related to the study drug
- Probably: The AE is likely related to the study drug
- Possible: The AE may be related to the study drug
- Unlikely: The AE is doubtfully related to the study drug
- Unrelated: The AE is clearly NOT related to the study drug

7.2 Serious Adverse Events

An AE is considered "serious" if in the opinion of the investigator it is one of the following outcomes:

- Fatal
- Life-threatening
- Constitutes a congenital anomaly or birth defect
- A medically significant condition (defined as an event that compromises subject safety or may require medical or surgical intervention to prevent one of the three outcomes above).
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant incapacity or substantial disruption to conduct normal life functions.

7.2.1 Reporting of SAEs

Any reactions that is reportable (serious, unexpected and related/possibly related to the study) must be reported using the FDA #3500 MedWatch form and the Duke University Health System (DUHS) Institutional Review Board (IRB) Office SAE form. All other side effects and toxicities will be recorded in the patient's research chart or documented on the computer record for the patient.

7.3 Special Warnings and Precautions

Specific plan in the event of microbial contamination of infused cellular product:

Product sterility testing will include a STAT gram stain which will have to be negative prior to release for infusion. A 14 day culture will be performed by our microbiology laboratory as per FDA requirements. In addition, per the SOP of sterility testing, cultures will be maintained for 5 days in our stem cell laboratory though typically the product is infused fresh the day of collection or the following day. In the event that a culture becomes positive, the lab notifies the treating physician immediately who in turn notifies the patient and the treating staff. Patients will be evaluated within the same day of notification of a positive culture by the treating team for any infection related incidents and treated appropriately. The evaluation will include a minimum of physical examination, vital signs and blood culture.

In the event of this occurrence, this will be considered an SAE and reported in an expedited manner as above for other serious adverse events if there is a change in the health status of the patient or requiring initiation of antimicrobials. Microbial contamination will otherwise be reported to the IND as an adverse event amendment submission within 7 days of occurrence.

Investigation plan in case of cellular microbial contamination:

In the event that a product has a positive culture, all reasonable efforts will be undertaken to detect the possible source. For potential donor source, donor notification and return to clinic or primary care physician for examination if there are any signs or symptoms of infection will be recommended. Examination of the chain of custody log to ensure proper transit of the product and review of the exact procedure with the laboratory technician who performed the cell selection to investigate any potential difficulties that arose (tubing malfunction, bag leakage, etc). If two such events are noted in the this

study without a correctable cause being found after investigation, the trial will be closed to accrual until the Duke cryo lab staff assigned to this study has been inserviced by a company representative from Miltenyi and the above issues are reviewed. Staff assigned and undergoing this additional education will be logged with a copy in the regulatory files for documentation. Upon reopening accrual, increased point of care testing for sterility will be added to our SOP at the following points:

- In the clinic after collection and prior to transmission to cryo-lab
- In the cryo-lab just prior to putting the product on the Miltenyi device
- in the cryo lab after completion of manipulation on the device and before transmission to the clinical lab for infusion
- In the clinic just prior to infusion into the patient

7.4 Safety Oversight Committee (SOC)

The Duke Cancer Institute SOC is responsible for annual data and safety monitoring of DUHS sponsor-investigator phase I and II, therapeutic interventional studies that do not have an independent Data Safety Monitoring Board (DSMB). The primary focus of the SOC is review of safety data, toxicities and new information that may affect subject safety or efficacy. Annual safety reviews includes but may not be limited to review of safety data, enrollment status, stopping rules if applicable, accrual, toxicities, reference literature, and interim analyses as provided by the sponsor-investigator. The SOC in concert with the DCI Monitoring Team (see Section 12.1 for Monitoring Team description) oversees the conduct of DUHS cancer-related, sponsor-investigator greater-than-minimal-risk intervention studies that do not have an external monitoring plan, ensuring subject safety and that the protocol is conducted, recorded and reported in accordance with the protocol, standing operating procedures (SOPs), Good Clinical Practice (GCP), and applicable regulatory requirements.

8 QUALITY CONTROL AND QUALITY ASSURANCE

8.1 Monitoring

The Duke Cancer Institute (DCI) Monitoring Team will conduct monitoring visits to ensure subject safety and to ensure that the protocol is conducted, recorded, and reported in accordance with the protocol, standard operating procedures, good clinical practice, and applicable regulatory requirements. As specified in the DCI Data and Safety Monitoring Plan, the DCI Monitoring Team will conduct routine monitoring after the third subject is enrolled, followed by annual monitoring of 1 – 3 subjects until the study is closed to enrollment and subjects are no longer receiving study interventions that are more than minimal risk.

Additional monitoring may be prompted by findings from monitoring visits, unexpected frequency of serious and/or unexpected toxicities, or other concerns and may be initiated upon request of DUHS and DCI leadership, the DCI Cancer Protocol Committee, the Safety Oversight Committee (SOC), the sponsor, the Principal Investigator, or the IRB. All study documents must be made available upon request to the DCI Monitoring Team and other authorized regulatory authorities, including but not limited to the National Institute of Health, National Cancer Institute, and the FDA. Every reasonable effort will be made to maintain confidentiality during study monitoring.

8.2 Audits

The Duke University Office of Audit, Risk and Compliance - Human Subjects Research Compliance (HSRC) office may conduct confidential audits to evaluate compliance with the protocol and the principles of GCP. The PI agrees to allow the HSRC auditor(s) direct access to all relevant

documents and to allocate his/her time and the time of the study team to the HSRC auditor(s) in order to discuss findings and any relevant issues.

HSRC audits are designed to protect the rights and well-being of human research subjects. HSRC audits may be routine or directed (for cause). Routine audits are selected based upon risk metrics generally geared towards high subject enrollment, studies with limited oversight or monitoring, Investigator initiated Investigational Drugs or Devices, federally-funded studies, high degree of risk (based upon adverse events, type of study, or vulnerable populations), Phase I studies, or studies that involve Medicare populations. Directed audits occur at the directive of the IRB or an authorized Institutional Official.

HSRC audits examine research studies/clinical trials methodology, processes and systems to assess whether the research is conducted according to the protocol approved by the DUHS IRB. The primary purpose of the audit/review is to verify that the standards for safety of human subjects in clinical trials and the quality of data produced by the clinical trial research are met. The audit/review will serve as a quality assurance measure, internal to the institution. Additional goals of such audits are to detect both random and systemic errors occurring during the conduct of clinical research and to emphasize "best practices" in the research/clinical trials environment.

8.3 Data Management and Processing

8.3.1 Case Report Forms (CRFs)

An electronic CRF maintained in a Microsoft Office Access database will be the primary data collection document for the study. The CRFs will be updated in a timely manner following acquisition of new source data. Only approved study staff (data coordinators), are permitted to make entries, changes, or corrections in the CRF.

An audit trail will be maintained automatically by the electronic CRF management. Designated personnel will complete user training, as required or appropriate per regulations.

8.3.2 Data Management Procedures and Data Verification

Designated personnel using the electronic CRF will have access based on their specific roles in the protocol. Regulatory and nursing coordinators will have read-only access. Data Coordinators will have edit access.

Completeness of entered data will be checked automatically by the eCRF system, and users will be alerted to the presence of data inconsistencies. Additionally, the data manager and/or data coordinator back-up will cross-reference the data to verify accuracy. Missing or implausible data will be highlighted for the PI requiring appropriate responses (i.e. confirmation of data, correction of data, completion or confirmation that data is not available, etc.).

The database will be reviewed and discussed prior to database closure, and will be closed only after resolution of all remaining queries. An audit trail will be kept of all subsequent changes to the data.

8.3.3 Study Closure

Following completion of the studies, the PI will be responsible for ensuring the following activities:

- Data clarification and/or resolution

- Accounting, reconciliation, and destruction/return of used and unused study drugs
- Review of site study records for completeness
- Shipment of all remaining laboratory samples to the designated laboratories

9.0 ADMINISTRATIVE AND ETHICAL CONSIDERATIONS

9.1 Regulatory and Ethical Compliance

This protocol was designed and will be conducted and reported in accordance with the International Conference on Harmonization (ICH) Harmonized Tripartite Guidelines for Good Clinical Practice, the Declaration of Helsinki, and applicable federal, state, and local regulations.

9.2 DUHS Institutional Review Board and DCI Cancer Protocol Committee

The protocol, informed consent form, advertising material, and additional protocol-related documents must be submitted to the DUHS Institutional Review Board (IRB) and DCI Cancer Protocol Committee (CPC) for review. The study may be initiated only after the Principal Investigator has received written and dated approval from the CPC and IRB.

The Principal Investigator must submit and obtain approval from the IRB for all subsequent protocol amendments and changes to the informed consent form. The CPC should be informed about any protocol amendments that potentially affect research design or data analysis (i.e. amendments affecting subject population, inclusion/exclusion criteria, agent administration, statistical analysis, etc.).

The Principal Investigator must obtain protocol re-approval from the IRB within 1 year of the most recent IRB approval. The Principal Investigator must also obtain protocol re-approval from the CPC within 1 year of the most recent IRB approval, for as long as the protocol remains open to subject enrollment.

9.3 Informed Consent

The informed consent form must be written in a manner that is understandable to the subject population. Prior to its use, the informed consent form must be approved by the IRB.

The Principal Investigator or authorized key personnel will discuss with the potential subject the purpose of the research, methods, potential risks and benefits, subject concerns, and other study-related matters. This discussion will occur in a location that ensures subject privacy and in a manner that minimizes the possibility of coercion. Appropriate accommodations will be made available for potential subjects who cannot read or understand English or are visually impaired. Potential subjects will have the opportunity to contact the Principal investigator or authorized key personnel with questions, and will be given as much time as needed to make an informed decision about participation in the study.

Before conducting any study-specific procedures, the Principal Investigator must obtain written informed consent from the subject or a legally acceptable representative. The original informed consent form will be stored with the subject's study records, and a copy of the informed consent form will be provided to the subject. The Principal Investigator is responsible for asking the subject whether the subject wishes to notify his/her primary care physician about participation in the study. If the subject agrees to such notification, the Principal Investigator will inform the subject's primary care physician about the subject's participation in the clinical study.

9.4 Study Documentation
Study documentation includes but is not limited to source documents, case report forms (CRFs), monitoring logs, appointment schedules, study team correspondence with sponsors or regulatory bodies/committees, and regulatory documents that can be found in the DCI-mandated "Regulatory Binder", which includes but is not limited to signed protocol and amendments, approved and signed informed consent forms, FDA Form 1572, CAP and CLIA laboratory certifications, and clinical supplies receipts and distribution records.

Source documents are original records that contain source data, which is all information in original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source documents include but are not limited to hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate copies, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories and at medico-technical departments involved in the clinical trial. When possible, the original record should be retained as the source document. However, a photocopy is acceptable provided that it is a clear, legible, and an exact duplication of the original document.

A case report form (CRF) (please indicate whether a paper or electronic CRF will be used) will be the primary data collection document for the study. The CRFs will be updated within two weeks of acquisition of new source data. Only approved study staff (data coordinators), are permitted to make entries, changes, or corrections in the CRF. For paper CRFs, errors will crossed out with a single line, and this line will not obscure the original entry. Changes or corrections will be dated, initialed, and explained (if necessary). The Principal Investigator or authorized key personnel will maintain a record of the changes and corrections. For electronic CRFs, an audit trail will be maintained by the electronic CRF management system (please indicate what eCRF management system is being used).

9.5 Privacy, Confidentiality, and Data Storage
The Principal Investigator will ensure that subject privacy and confidentiality of the subject's data will be maintained. Research Data Security Plans (RDSPs) will be approved by the appropriate institutional Site Based Research group.

To protect privacy, every reasonable effort will be made to prevent undue access to subjects during the course of the study. Prospective participants will be consented in an exam room where it is just the research staff, the patient and his family, if desired. For all future visits, interactions with research staff (study doctor and study coordinators) regarding research activities will take place in a private exam room. All research related interactions with the participant will be conducted by qualified research staff who are directly involved in the conduct of the research study.

To protect confidentiality, subject files in paper format will be stored in secure cabinets under lock and key accessible only by the research staff. Subjects will be identified only by a unique study number and subject initials. Electronic records of subject data will be maintained using a dedicated database (Microsoft Access), which is housed in an encrypted and password-protected DCI file server (\\cancerlan6\adultbmt_pro). Access to electronic databases will be limited to staff of the division of Cellular Therapy. The security and viability of the IT infrastructure will be managed by the DCI and/or Duke Medicine.

Upon completion of the study, research records will be archived and handled per DUHS HRPP policy.

Subject names or identifiers will not be used in reports, presentations at scientific meetings, or publications in scientific journals.

9.6 Data and Safety Monitoring

Data and Safety Monitoring will be performed in accordance with the DCI Data and Safety Monitoring Plan. For a more detailed description of the DSMP for this protocol, refer to Section 8.

9.7 Protocol Amendments

All protocol amendments must be initiated by the Principal Investigator and approved by the IRB prior to implementation. IRB approval is not required for protocol changes that occur to protect the safety of a subject from an immediate hazard. However, the Principal Investigator must inform the IRB and all other applicable regulatory agencies of such action immediately.

Though not yet required, the CPC should be informed about any protocol amendments that potentially affect research design or data analysis (i.e. amendments affecting subject population, inclusion/exclusion criteria, agent administration, etc.).

9.8 Records Retention

The Principal Investigator will maintain study-related records for the longer of a period of:

- at least two years after the date on which a New Drug Application is approved by the FDA (if an IND is involved)
- at least two years after formal withdrawal of the IND associated with this protocol (if an IND is involved)
- at least six years after study completion (Duke policy)

Appendix I: Toxicity Forms

Table 8: Grading GVHD

Stage	Skin	Liver	Gut
1	Rash on <25% of skin	Bilirubin 2.0-3.0 mg/dl	Diarrhea >500 ml/day or persistent nausea
2	Rash on 25-50% of skin	Bilirubin 3.1-6.0 mg/dl	Diarrhea >1000 ml/day
3	Rash on >50% of skin	Bilirubin 6.1-15.0 mg/dl	Diarrhea >1500 ml/day
4	Generalized erythroderma with bullous formation	Bilirubin >15 mg/dl	Severe abdominal pain with or without ileus
Grade			
I	Stage 1-2	None	None
II	Stage 3 or	Stage 1 or	Stage 1
III	-	Stage 2-3 or	Stage 2-4
IV	Stage 4 or	Stage 4	-

(Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transplant. 1995 Jun;15(6):825-8.)

Common Toxicity Criteria Version 4.0

http://ctep.cancer.gov/reporting/ctc.html

Appendix II
ECOG PERFORMANCE STATUS SCALE

ECOG Scale*	ECOG Description
0	Fully active, able to carry on all pre-disease activities without restriction.
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature e.g. light house work office work.
2	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	Capable of only limited self care, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any self care. Totally confined to bed or chair
Dead	

Appendix III: Patient Eligibility Checklist

Desired answer	Answer	Date determined	**Eligibility Criteria**
Inclusion criteria			
yes			Patients with hematologic diseases who have undergone allogeneic transplantation, using a 7-8/8 HLA-matched related or unrelated donor or 4-6/8 HLA-matched related donor. This may include patients with a mixed chimeric state or disease persistence or at high risk of relapse (these are patients who do not fit exclusionary principle 3 below).
yes			Performance status must be ECOG PS 0, 1, or 2
yes			Donor cellular engraftment of at least 2.5%
yes			<Grade 2 acute GVHD at time of the first NK cell-enriched DLI. Patients with treated acute GVHD must be on a stable dose of therapy (no increase in immunosuppressive therapy for the 2 weeks before planned NK cell-enriched DLIs). The dosage/level of immunosuppressive therapy at the time of NK-DLIs should be no greater than 20 mg/kg of prednisone daily or mycophenolate 500 mg bid daily or cyclosporine with a target level of 200 ng/mL or tacrolimus with a target level of 10 ng/ml.
yes			Estimated survival of at least 8 weeks
yes			Age of >= 18 years
Yes			Females of childbearing potential should have a negative serum beta-HCG test within 48 hours of beginning DLI and/or DUK-CPG-01 unless contraception is used after initial testing.
Yes			Males must agree to use a medically acceptable form of birth control in order to be in this study and for 3 months after infusion
Exclusion criteria			
no			Pregnant or lactating women
no			Patients with other major medical or psychiatric illnesses, which the treating physician feels, could seriously compromise tolerance to this protocol.
no			Patients likely to have a significantly better durable response to allogeneic transplant alone (better than 60% progression free longer than 2 years) includes: those with uncomplicated myeloproliferative diseases or hemoglobinopathies with over 50% T cell subset engraftment (assessed around 100 days post transplant); It is not anticipated that any such patients would be transplanted within our program, however those in first remission AML patients with good risk standard genetics or normal genetics with only either NPM1 or CEBPA mutations, first chronic phase CML without kinase gene mutations, follicular lymphoma patients in first remission who only required 1 regimen to attain remission all would be excluded from this protocol.

Appendix IV: Donor Eligibility Checklist

Desired answer	**Answer**	**Date determined**	**Eligibility Criteria**
4-8/8 HLA matched related donor			
yes			Potential donors under the age of 18 must have a single patient exemption approved by the IRB.
yes			Family members will be considered for donation if they do not have a history of known cardiac problem and do not have abnormal cardiac findings by physical examination. Those with a history of cardiac problems or abnormal cardiac findings by physical examination should undergo a stress evaluation or be evaluated by a cardiologist and deemed eligible to donate.
yes			Documented Bilirubin and hepatic transaminases < 2.5 x ULN
yes			Documented Adequate hematologic parameters including a hematocrit > 35% for males and 33% for females, white blood cell count of ≥3,000, and platelets ≥80,000.
yes			FACT labs and final test results available prior to infusion into the patient (copy of labs included in appendices). In the second donation from the donor, the FACT labs must be redrawn within 30 days of initiation of apheresis. Positive serologies are not repeated.
yes			Females of childbearing potential should have a negative serum beta-HCG test within 1 week of beginning apheresis.
8/8 HLA matched unrelated donor			
yes			Donor is an NMDP matched unrelated donor (Note: If yes, checking the above eligibility criteria is not needed).

Appendix V: Schedule of Events (This chart is a guide and full protocol takes precedence over this chart)

Table 9

	1st DLI[4]				2nd DLI[5] (if performed)				After the last DLI[9]				
NK cell-enriched DLI	**Pre[1]**	**Post 1day**	**Post 1M[10]**		**Pre[1]**	**Post 1day**	**Post 1M[10]**		**Post 3M[11]**	**Post 6M[11]**		**Post 12M[1] [1]**	**Q6 mos x 4[11]**
History and Physical	x		x		x		x		x	x		x	x
Blood tests for routine monitoring[2]	x		x		x		x		x	x		x	x
Serum Pregnancy testing[8]	x				x								
Assessment of disease[3]	x				x[7]				x	x		x	x
RFLP (blood or marrow)	x				x[6]				x	x		x	
Chimerism	x				x				x	x		x	
Immune Reconstitution	x	x	x		x	x	x		x	x		x	
Research blood Samples	x	x	x		x	x	x		x	x		x	
Stool collection	x		x		x		x		x	x		x	

[1] Screening, within 1-3 weeks prior to DLI

[2] Pre-infusion: CBC (with differential preferred), creatinine, AST, ALT, and total bilirubin, calcium, LDH, potassium, sodium, bicarbonate, chloride and glucose, albumin, phosphorous, magnesium and alkaline phosphatase. Post-infusion: CBC (with differential preferred), creatinine, AST, ALT, total bilirubin, and BUN

[3] Restaging will use standardized criteria and shall include a careful exam of the studies needed to detect the disease (PET, radiographs, immunos, marrow, molecular studies etc.). Restaging may be altered at the discretion of the transplant physician following the patient (who are all sub-investigators in this study) if the patient is felt to be progressing before these time points but the recommended restaging is q3 months for 1 year after the last NK-enriched DLI, q6 months for the next 2 years.

[4] The first NK cell-enriched DLI will be administered at 1-6 months post-transplant.

[5] The second NK cell-enriched DLI will be administered 1-3 months post the first infusion, in patients who have < grade II aGVHD at the time of infusion and have not had unacceptable toxicities (see 6.2.1 Definition of unacceptable toxicity) that are at least possibly related to the previous DLI and resolved to grade 1 or less. The second DLI will NOT be administered in patient with ≥grade III aGVHD at the time of infusion or unacceptable toxicities at least possibly related to the first infusion.

[6] Marrow does not need to be redrawn if the patient is in a complete remission based on marrow collected within 30 days of DLI.

[7] Scans do not need to be performed if the patient is in a complete remission based on scans performed within 30 days of DLI.

[8] For females of childbearing potential only.

[9] If the 2nd DLI is unable to be performed, follow-up will be from the time of 1st DLI.

[10] A window of +/- 2 days is allowed.

[11] A window of +/- 2 weeks is allowed.

Appendix VI: Research Lab Reference Sheet

Schedule of Events	Pre-DLI (pre-2nd DLI)	During Therapy (Day +1 and 1 month post-DLI)	3,6,and 12 mo after completing therapy for 1 year
NK Lysis Assay	60 cc G	60 cc G	60 cc G
Phenotype Flow Analysis	40 cc G	40 cc G	40 cc G
Cytokine	4 cc L	4 cc L	4 cc L
Total	104 cc: - 4 L -100 G	104 cc: - 4 L -100 G	104 cc: - 4 L -100 G

G = green (heparin), L = lavender (EDTA)

Lavender tubes: separate and freeze plasma

Green tubes: 100 cc ficoll/cryopreserve PBMCs at -80

**** Please remember****

- Alert lab staff of the patient's appointment 48 hours in advance
- Schedule early morning appointment for samples to be drawn

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