# A Pilot Study in the Treatment of Refractory BK Infections with Related Donor BK Specific Cytotoxic T-cells (CTLs) in Children, Adolescents and Adult Recipients – NYMC 590

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List of Abbreviations

|  |  |
| --- | --- |
| AdV | Adenovirus |
| Allo | Allogeneic |
| BMT | Bone marrow transplant |
| BK | Cytomegalovirus |
| CTL | Cytotoxic T lymphocytes |
| DLI | Donor lymphocyte infusion |
| EBV | Epstein-Barr virus |
| GVHD | Graft vs host disease |
| HSCT | Hematopoietic stem cell transplantation |
| PID | Primary immunodeficiency |
| SAE | Severe adverse event |
| SCID | Severe combined immunodeficiency |
| SOP | Standard operating procedures |

# Study Hypothesis

BK cytotoxic T cells (CTLs) manufactured with the Miltenyi CliniMACS Prodigy Cytokine Capture System will be safe and effective in decreasing specific viral load in children, adolescents and adults (CAYA) with refractory BK infection post Allogeneic Hematopoietic Stem Cell Transplantation (AlloHSCT), primary immunodeficiencies (PID), or post solid organ transplantation (SOT).

# Objectives

* 1. Primary Objectives
		1. To determine the safety of BK-specific T cells (CTLs)- in the treatment of children, adolescents and adults with refractory BK infection after allogeneic hematopoietic stem cell transplantation (HSCT), with primary immunodeficiencies or post SOT.

2.1.2 To estimate the efficacy of BK- specific T cells (CTLs) in the treatment of children, adolescents and adults with refractory BK infection after allogeneic HSCT, with primary immunodeficiencies or post SOT.

* 1. Secondary Objectives
		1. To determine the probability and severity of acute GVHD and chronic GVHD following BK CTL administration in children, adolescents and adults with refractory BK infection after AlloHSCT, with PID or post SOT.
		2. To estimate the persistence of third-party BK CTLs following BK CTL administration in children, adolescents and adults with refractory viral infection(s) after AlloHSCT, PID or post SOT.
		3. To determine the probability of 6 month and 1 year overall survival (OS) and viral- free survival (VFS) following BK CTL administration in children, adolescents and adults with refractory BK infection after AlloHSCT, with PID or SOT.
		4. To further investigate the genetic, proteomic and immunological properties of BK CTLs derived from the Miltenyi CliniMACS Prodigy Gamma-capture system.
		5. To quantitate specific cellular immune reconstitution and its correlation to antiviral response following BK CTL infusions.

# Background and Rationale

* 1. **BK Virus Infection in Transplant Recipients Background**

Polyomavirus BK, similar to other DNA viruses such as BK and EBV, establishes a latent state after primary infection.1,2 BK virus infects about 90% of the general population by 10 years of age and then remains dormant in the urothelial cells of the kidney and bladder.3,4 A competent immune response to BK virus involves cell- mediated mechanisms to prevent viral reactivation, cell lysis, and tissue damage.5 Clinical disease (hemorrhagic cystitis and/or direct nephropathy) is almost exclusively limited to patients receiving immunosuppression, such as solid organ (most notably kidney transplant) and hematopoietic stem cell transplant (HSCT) recipients.opulic, 2008 #20} However, there are also several reports of BK disease occurring in leukemia patients 6,7 and this has also occurred at the Children’s Hospital of Philadelphia. In kidney transplant recipients, BK virus infection typically manifests as nephropathy with a slowly increasing serum creatinine over time. In HSCT recipients, BK virus is typically associated with hemorrhagic cystitis of varying severity, from mild, asymptomatic hematuria to life threatening bleeding and obstruction in the bladder.

# BK virus infection and hemorrhagic cystitis after HSCT

BK viruria is common after HSCT and occurs even in the absence of hemorrhagic cystitis. 8 Up to 80% of HSCT recipients are noted to have BK viruria 9,10, but only 10- 25% of all patients develop clinically significant cystits.14 The mechanism by which BK virus infection leads to hemorrhagic cystitis in this subset of transplant patients is unknown, but may be related to immune-mediated bladder injury, GVHD, or later effects of conditioning chemotherapy, such as cyclophosphamide.11,13,14 Hemorrhagic cystitis is a significant complication after HSCT and is associated with prolonged hospitalization, urinary tract obstruction, and possibly death.9-11,15 Aggressive hydration and MESNA with cyclophosphamide administration have decreased this as a risk of cystitis. However, late-onset cystitis (>72 hours after conditioning) has become the more common post-transplant bladder complication. 11,14,16 Potential risk factors for late- onset cystitis include the type of conditioning chemotherapy, timing of engraftment, development of graft versus host disease (GVHD), presence of BK virus infection in the blood or urine, and other viral infections. 11,13,16 After HSCT, 80% of recipients with hemorrhagic cystitis have BK viruria. 9,15 There are no screening guidelines for BK virus

infection after HSCT.17,18 Cesaro et al. prospectively studied 107 pediatric patients who received an allogeneic-HSCT, and found that a plasma BKV DNA viral load of 1,000 copies/mL (1 x 103) had a sensitivity of 100% and specificity of 86% to predict hemorrhagic cystitis.9 However, Ghosh et al. found no plasma level of BK virus that was predictive of development of hemorrhagic cystitis in 224 allogeneic HSCT recipients (on multivariate analysis, only cord HSCT was found to be independently associated with developing BK hemorrhagic cystitis). 19 It is therefore unknown at this time what level of BK virus is predictive of hemorrhagic cystitis.

# BK virus infection and nephropathy after HSCT

Kidney injury is a significant complication of HSCT. In addition, chronic kidney disease (CKD) occurs in at least 20% of children after HSCT, although the exact prevalence is unknown.20,21 Adults surviving after HSCT have a risk of end stage kidney disease 16 times higher than the general population.22 Compared to adults, children may experience an even greater long-term benefit in terms of years of life gained from the prevention of CKD-associated morbidity and mortality. 21,23 Despite a BK viremia prevalence of at least 15%, it is unclear if viremia contributes to CKD in HSCT survivors as it does in kidney transplant recipients.10,15,24,25

Data on the contribution of BK viremia to intrinsic kidney injury after HSCT has been primarily limited to case reports.26-30 One study in adults receiving an HSCT found BK viremia was associated with kidney injury (increased peak creatinine).25 Among 21 pediatric HSCT recipients having BK virus PCR testing at Cincinnati Children’s performed for symptoms of cystitis, children with peak BK viremia ≥10,000 copies/ml during the first year had a significantly higher peak serum creatinine compared to those with lower viremia.*31* Therefore, it is plausible that BK virus infection can be associated with nephropathy, and not just hemorrhagic cystitis, in HSCT recipients.

# BK virus infection and nephropathy after solid organ transplantation

BK virus is known to be associated with direct kidney injury (nephropathy) in patients who have received a kidney transplant.32 Several smaller reports have also identified an association between BK virus infection and kidney disease in patients who have received a non-renal solid organ transplant (heart, liver, lung). 33-35 BK viruria (viral detection in the urine) occurs in about 30% of patients after renal transplant, BK viremia in about 10%, and BK nephropathy in 2-5% of recipients.36 Kidney transplant consensus guidelines support routine screening for BK viremia after kidney transplant in an effort to detect BK virus early, potentially allowing intervention before kidney damage becomes irreversible.37 BK viremia has higher positive predictive value for biopsy-proven

nephropathy than BK viruria.36 Reduction in immunosuppression, when feasible, is the current standard of care in the treatment of BK nephropathy and BK viremia in patients after kidney transplant.38,39 However, given the risk of rejection with lowering immunosuppression, not all patients are able to have their immunosuppression reduced. Furthermore, some patients will continue to have BK virus replication and evidence of tissue injury (renal biopsy) despite lowering of immunosuppression. There is a need for novel, targeted therapeutic strategies in patients with BK virus infection after kidney transplant.

# Immunological control of BK virus infection

The BK viral genome is composed of 3 sections: a non-coding promoter region, early regulatory proteins (large T and small T antigens), and late structural capsid proteins (VP1, VP2, VP3) and the agnoprotein (whose role is unknown).5 While the immunodominant BK virus antigen is not known, most studies in kidney transplant recipients have focused on VP1 and the large T antigen.40,41 Immune responses to the VP capsid proteins may occur before responses to the regulatory proteins (large T and small T).

After primary infection, the host immune response involves generation of neutralizing antibodies and memory T-cells.42-45 It is unknown if neutralizing antibodies provide complete protection against future disease as reactivation of latent infection can occur in previously seropositive kidney transplant and HCT recipients.5,8,46 Antibody titers may simply be a marker of a functioning T-cell response. Seronegative pediatric kidney transplant recipients are at higher risk of post-transplant BK virus nephropathy.47,48Most adults are BK IgG seropositive, and BK virus-related disease is believed to occur through viral reactivation.4 However, younger, seronegative children may have an increased risk of disease from primary infection.48 Additionally, BK virus exists as at least 4 distinct genotypes, making it possible that primary infection could occur with a different genotype in a previously seropositive recipient.49-51

Only a few studies have assessed BK-specific T-cell responses after HSCT, primarily in patients with hemorrhagic cystitis and viruria.5,51 However, the association between BK viremia, the kinetics of the immune response, and subsequent kidney injury has not been studied after HSCT.

# Current Therapy for BK Infection

There is no effective therapy for BK infection. Cidofovir intravenously or intravesicularly has been used, with response rates of approximately 60%52, but no decrease noted in

BK viral load. 53 There are also ongoing studies of ciprofloxacin and leflunomide, but little data to support these drugs as effective standard therapy.

# Conclusions

BK virus infection is common in immunosuppressed hosts and is associated with hemorrhagic cystitis and intrinsic kidney injury in HCT and solid organ transplant recipients. Current treatments have not been systematically assessed and are associated with significant toxicity without proven efficacy. It is likely that similar to other DNA viruses, cell-mediated immune mechanisms are responsible for controlling BK virus infection and preventing/limiting disease in susceptible hosts. Therefore, the potential for infusing BK virus-specific T-cells as a prophylactic or treatment strategy has great promise.

# 1.2 Immunologic therapy of viral infections

Various methods for immunologic treatment of viral infections/reactivations after HSCT have been explored. Successful adoptive transfer of cytotoxic T-cell clones in bone marrow transplant recipients have been published as early as 1992 54 Initially, unselected donor lymphocytes (DLIs) were utilized to treat viral infections, but this therapy was associated with a higher incidence of GVHD in recipients of unrelated donor or haploidentical transplants due to the high number of alloreactive T cells. A recent review summarized the newer methodologies and results of virus-specific T cell therapy. 54 Each offers advantages and each is associated with different challenges. 55 Virus-specific T cell therapy requires a defined immunogenic antigen and an antigen presenting cell that can present to T cells with appropriate co-stimulatory signals. One currently established method for the generation of tri-specific cytotoxic T lymphocytes (CTL) for BK, EBV and AdV uses repetitive re-stimulation of peripheral blood mononuclear cells with EBV-LCLs (lymphocyte cell lines) transduced with an Ad5f35pp65 vector.56 However, this method is limited by the time to develop these cells, which may take up to 14 weeks, and the production process. Lymphocytes have to be kept in extended cell culture with repeated feeding and weekly stimulations with

antigen-presenting cells (APCs).

Tetramer selection is another method that has been used successfully. This is a GMP compliant strategy in which virus-specific T cells from bulk donors’ T lymphocytes are selected by tetramer selection.57,58 T cells are rapidly available, and the selection process does not require antigen presenting cells, exogenous cytokines or extended ex

vivo manipulation. However, tetramer-mediated selection only selects T cells specific for a single HLA-restricted epitope of a single virus, and is generally only available for donors with the most common HLA types. Focusing the antiviral response leaves the patient vulnerable to antigenic escape.

# Viral Infections Post Solid Organ Transplantation

Viral infections for recipients of solid organ transplants (SOT) are extremely challenging to treat and remain a significant cause of morbidity and mortality.59 These infections, including EBV, CMV, adenovirus and BK, can occur *de novo*, via transmission from the transplanted organ, or reactivation from latent virus. Complications from these viral infections include development of actual disease, graft loss and organ dysfunction.

While decreased immune suppression may help decrease viral load, this intervention may not be possible without increased risk of rejection, and anti-viral antibiotics may not be tolerated or effective. Manufacture of cytotoxic T lymphocytes (CTLs) with the CliniMACS Cytokine Capture System (CCS) directed against these viruses may decrease morbidity and mortality, and improve outcomes.

Epstein-Barr virus is associated with post-transplant lymphoproliferative disorder (PTLD) and tumor formation. It is the most common non-skin cancer malignancy following SOT, and is fatal in up to 60% of patients. The incidence of PTLD is more common in pediatric patients, as many are seronegative prior to transplant with an EBV positive organ.60-63 Incidence varies among transplanted organ, with intestine, multi- organ and lung being more common. Therapy includes decreased immune suppression, rituximab and chemotherapy. However, for refractory or recurrent disease, EBV CTLs have been effective in treating PTLD post SOT.64-67

CMV infection is one of the most common infections after SOT, and may result in significant morbidity, mortality, and graft loss.68,69 It has been associated with nephropathy and allograft loss after renal transplant, accelerated hepatitis C infection after liver transplant, allograft vasculopathy in cardiac transplant, and bronchiolitis obliterans after lung transplant. Anti-viral therapy may be limited in efficacy due to poor tolerance, development of resistance.70

Adenovirus (ADV) infections are more common in pediatric SOT recipients. 71,72 In recipients of liver transplants, ADV related hepatitis and pneumonia are associated with a high mortality rate of 43% and 75% respectively. Antibiotic therapy is limited to cidofovir, and its nephrotoxicity limits its use. ADV CD4+ and CD3+ T cells are required for complete antiviral protection, and the Miltenyi CCS manufactures both types.

BK virus nephropathy occurs in up to 10% of renal transplant recipients and can result in significant renal dysfunction and graft loss. There are no effective anti-viral agents for BK, and CTLs may decrease the risk of graft loss and BK nephropathy.

Most data regarding CTLs for SOT comes from EBV directed therapy manufactured by other methods.64-66 Neither GVHD nor graft rejection have been described. We have not seen GVHD or rejection in patients post HSCT who received vCTLs manufactured with the Miltenyi CCS. At the Children’s Hospital of Philadelphia, we have given 4 infusions of haploidentical EBV CTLs manufactured with the Miltenyi CCS to a patient post kidney transplant, and this patient had no rejection, GVHD or other problems post infusions. The infusions were effective in resolving his EBV PTLD. The possibility of immunotherapy with vCTLs increases therapeutic options in these SOT patients with viral infections.

# CliniMACS Gamma Interferon Cytokine Capture System

* + 1. **Background**

This study is using the transfer of directly enriched virus-specific T cells and has been under development for >10 years. The CliniMACS Cytokine Capture System (IFN- ) allows rapid direct enrichment of virus-specific CD4+ and CD8+ T cells after incubation with the respective viral antigens. 73 This method exploits the natural mechanism that antigen-specific memory T cells produce IFN-gamma upon incubation with the specific antigen. The successful enrichment of virus-specific T cells using the CliniMACS IFN- Cytokine Capture System after incubation has been well established in pre-clinical studies. The CliniMACS Prodigy, recently developed by Miltenyi Biotec, allows for fully automated generation of multivirus-specific T cells for adoptive T cell therapy.21,74 Kinetics of the IFN-gamma response, cytotoxicity, alloreactivity and in-vitro expansion of the enriched cells have been investigated and analyzed thoroughly. 75,76The successful generation of multi-virus specific T cells after simultaneous incubation with several MACS GMP PepTivator peptide pools has also been demonstrated.76Isolation of both CD8+ and CD4+ specific T cells help prevent immune escape of these viruses.77-79

Clinical results and safety of the transfer of virus-specific T cells isolated and selected as described above with the CliniMACS Plus system are available. Patients have been treated with BK, adenovirus or EBV infections post HSCT. 57,80-82

The selection process is the CliniMACS Cytokine Capture System (IFN ) which allows rapid direct enrichment of virus-specific CD4+ and CD8+ T cells after incubation with the respective viral antigens.73,83 The method was first described in 199984 and exploits the natural mechanism that antigen-specific memory T cells produce interferon-gamma upon incubation with the specific antigen. In the first step of the selection process, cells are incubated with specific viral antigens triggering the intracellular production of IFN (MACS GMP PepTivator® Peptide Pools). They are then labeled with two different IFN

-specific antibodies in a stepwise procedure. The first binding step uses the CliniMACS IFN -gamma Catchmatrix Reagent, and for the second binding step the CliniMACS IFN Enrichment Reagent is used. The Catchmatrix Reagent forms a cytokine affinity matrix on the cell plasma membrane which then will ‘trap’ all cytokine subsequently produced by the cells upon specific stimulation.85 The Enrichment Reagent then binds to the trapped cytokine, thus enhancing the signal. The enrichment antibody is conjugated to super-paramagnetic particles and final selection of the antibody/cell complexes is performed using the long established MACS® technology (‘Magnetic Assisted Cell Sorting’).82 The successful enrichment of virus-specific T cells using the CliniMACS® IFN Cytokine Capture System after incubation with PepTivator® peptide pools as viral antigens has been well established in preclinical studies. The CliniMACS® Prodigy, which has been developed recently by Miltenyi Biotec GmbH, allows the fully automated generation of multivirus-specific T cells for adoptive T-cell therapy.74

Kinetics of the IFN response, cytotoxicity, alloreactivity and in vitro-expansion of the enriched cells have been investigated and analyzed thoroughly. 78,86,87 The successful generation of multivirus-specific T cells after simultaneous incubation with several MACS GMP PepTivator peptide pools has also been demonstrated. 88

The CliniMACS® Prodigy which will be used in this study is a newly developed system for the fully automated selection and isolation of virus-specific T cells. The safety and efficacy of the virus-specific T cells isolated and selected with this method have been described in several publications.73,74,76The virus-specific T cells were used for patients with refractory AdV89, BK 90,91, or EBV infections 81 after HSCT.

# Clinical Studies using BK Cytotoxic T-Lymphocytes (CTLs)

There is limited experience with the safety and efficacy of BK-CTLs. Papadopoulou et al reported the results of BK-CTLs in 7 patients that resulted in 5 CR, 1 PR and 1 NR and

was well tolerated.92 Pello et al reported the use of the gamma captor system in manufacturing BK-CTLs in one patient that resulted in a CR and was well tolerated. 93 Tzannou reported in 20 patients with refractory BK infection the use of BK-CTLs manufactured by ex-vivo expansion and demonstrated 6 CR and 14 PR and was in general well tolerated.94 Baugh et al recently reviewed the infusion of viral CTLs in HSCT recipients highlighting the safety and efficacy and life-saving nature of this approach.95 Although somewhat unrelated, Muftuoglu et al infused BK CTLs successfully in 2 patients with progressive multifocal leukoencephapathy. 96

Cytokine release syndrome has not been reported in any infusion of viral CTLs generated by the Cytokine Capture System.

# Experimental Design

* 1. HLA Matched Related Donors: BK specific CTLs (2.5 x 104 CD3/kg) infused intravenously on day 0 and may be additionally reinfused at a minimum of every two weeks (depending on safety and efficacy) for a maximum of five total infusions (maximum 12.5 x 104 CD3/kg).
	2. HLA Mismatched Related Donors: BK specific CTLs (0.5x104 CD3/kg) infused intravenously on day 0 and may additionally be reinfused at a minimum of every two weeks (depending on safety and efficacy) for a maximum of five total infusions (maximum 2.5 x 104 CD3/kg).

# Patient and Donor Eligibility

* 1. **Patient Eligibility**
		1. Patients with refractory BK infection post allogeneic HSCT or with primary immunodeficiencies or post solid organ transplantation (SOT) with either
			+ Increasing urine and/or plasma BK RT-PCR DNA (from baseline) after 7 days or persistent quantitative qRT-PCR DNA copies after 14 days despite two weeks of appropriate anti-viral therapy

AND/OR

* + - * Medical intolerance to anti-viral therapies including:

->2 renal toxicity with cidofovir or other > grade 2 toxicities secondary to cidofovir

And/or

-known resistance to cidofovir

5.1.2. Consent*:* Written informed consent given (by patient or legal representative) prior to any study-related procedures.

* + 1. Performance Status > 30% (Lansky < 16 yrs and Karnofsky > 16 yrs)
		2. Age: 0.1 to 30.99 years (Cohort 1)

Age: 31 to 79.99 years (Cohort 2)

* + 1. Females of childbearing potential with a negative urine pregnancy test at study entry only.

# Donor Eligibility

5.2.1 Related donor available with a T-cell response BK MACS® PepTivators. As defined in Appendix II, B., 8.2, the donor is considered suitable if the percentage of

IFN

T cells is >0.01% after stimulation with the screening BK PepTivators.

a. Third Party Allogeneic Donor: If original donor is not available or does not have

+

a T-cell response to BK MACS® PepTivators: **third party related allogeneic donor** (family donor > 3 HLA A, B, DR match to recipient) with IgG positive to BK and/or a T- cell response to the BK MACS® PepTivator .

AND

Allogeneic donor disease screening is complete similar to hematopoietic stem cell donors (Appendix 1).

AND

Obtained informed consents by donor or donor legally authorized representative prior to donor collection.

# Patient exclusion criteria:

A patient meeting any of the following criteria is not eligible for the present study:

* + 1. Patient with acute GVHD > grade 2 or severe chronic GVHD at the time of BK CTL infusion
		2. Patient receiving steroids (>0.5 mg/kg prednisone equivalent) at the time of BK CTL infusion
		3. Patient treated with donor lymphocyte infusion (DLI) within 4 weeks prior to BK CTL infusion
		4. Thymoglobulin (ATG) or Alemtuzumab within 30 days
		5. Patient with poor performance status determined by Karnofsky (patients

>16 years) or Lansky (patients ≤16 years) score ≤30%

* + 1. Concomitant enrollment in another experimental clinical trial investigating the treatment of refractory BK infection.
		2. Any medical condition which could compromise participation in the study according to the investigator’s assessment
		3. Known AIDS/uncontrolled HIV infection
		4. Female patient of childbearing age who is pregnant or breast-feeding or not willing to use an effective method of birth control during study treatment.
		5. Known hypersensitivity to iron dextran
		6. Patients unwilling or unable to comply with the protocol or unable to give informed consent.
		7. Known human anti-mouse antibodies

# Treatment

* 1. **Study Overview**

This open-label, phase I/II clinical trial will assess the safety and efficacy of BK-specific CTLs isolated from whole blood or leukapheresis products. The BK-specific CTLs will be generated automatically by the CliniMACS® Prodigy using the CliniMACS Cytokine Capture System (IFN-gamma) after incubation with MACS GMP PepTivator® Peptide Pools of pp65 (BK).

Patients will be assigned to a cohort based on age:

Cohort 1 will enroll patients up to age 30.99 years. Cohort 2 will enroll patients 31 to 79.99 years.

* 1. **BK CTLs: Dose and Administration:**

**\*ALL PATIENTS MUST RECEIVE the FIRST CTL INFUSIONS AS AN INPATIENT AND BE MONITORED INPATIENT FOR ADVERSE EVENTS FOR AT MINIMUM 24 HOURS FOLLOWING the CTL INFUSION\***

**The patient maybe discharged if afebrile and has normal heart rate, respiratory rate, blood pressure and is on room air. The patient will need to be seen daily for 5 more days as an outpatients to be observed for toxicity or adverse events.**

**If subsequent CTL infusions are needed, no adverse effects were experienced with the first CTL infusion and infusion criteria are met (see protocol sections 6.2.4 and 6.5) they may be administered outpatient per physician discretion monitoring per section 6.4 and observation up to 4 hours after the start of the infusion.**

* + 1. Suspension of BK-specific T cells in 10 mL of 0.9% NaCl with human serum albumin (HSA) given by IV bolus injection
		2. HLA-identical related donors: Dose 2.5 × 104 CD3/kg recipient weight.
		3. HLA mismatched related donors (mismatch at 1-5 antigens/alleles) Dose

0.5 × 104 CD3/kg recipient weight.

* + 1. Additional doses of BK CTLs a minimum of every 2 weeks (maximum 5 doses total)
			1. - If recipients fail to respond to the first dose of BK CTLs (qRT-PCR over the institutional level of upper normal) and have no Grade II acute or chronic GVHD and no persistent toxicities related to the past CTL infusions
		2. Dose in HLA related matched donors: Max dose with each infusion 2.5 x 104 CD3/kg until a max combined dose of 12.5 x 104 CD3/kg (5 total doses)
		3. Dose in HLA related Mismatched donors: Max dose with each infusion of 0.5 x 104 CD3/kg with a max combined dose of 2.5 x 104 CD3/kg (5 total doses).

# Concomitant Medications

* + 1. Antiviral Treatment

All patients will be additionally treated with antiviral chemotherapy as per local institutional standards: cidofovir. Change of second-line therapy to any of the above mentioned medications according to the investigator’s assessment is allowed.

Prophylactic and/or systemic treatment with other antivirals are allowed throughout the study.

* + 1. Prohibited medication and procedures

During the study, treatment with other investigational anti-BK agents and treatment with donor lymphocyte infusions (DLIs) are prohibited in patients treated with the BK-CTLs until Week 12 and will be considered off study if new systemic antiviral therapy is initiated

* 1. **Vital Signs**: temperature, blood pressure and heart rate will be obtained at 15, 30 60 and 120 minutes after each BK CTL infusion.
	2. **CTL infusion will be held** if any one of the following is present:
1. ATG or alemtuzumab within 30 days
2. DLI within last 30 days
3. >grade II AGVHD
4. Patient receiving steroids (>0.5 mg/kg prednisone equivalent) at the time of CTL infusion
5. Any dose limiting toxicity event (see 18.1.3) possibly, probably or definitely related to any BK CTL prior infusion
6. Any grade 3-5 infusion-reaction, as graded by the NCI CTCAE v5.0, possibly, probably or definitely related to any BK CTL prior infusion
7. Recipient seroconversion to any FDA-listed relevant communicable diseases which upon investigation, is determined to be caused or potentially caused by the BK CTL infused.
8. Recipient septicemia is determined to be caused or potentially caused by contaminated BK CTL infusion
9. Performance status less than 30%.

If none of these criteria exist BK-specific CTLs will be administered. Patients may be premedicated with diphenhydramine up to 1mg/kg (max 50 mg) IV and acetaminophen 10mg/kg (max 650 mg) PO 30-60 minutes prior to infusion. **Premedication with corticosteroids is prohibited.**

* 1. Management of Toxicity probably or definitely related to BK CTLs

Patients with grade III-IV infusional toxicity probably or definitely attributable to BK- specific CTLs- will receive solumedrol or prednisone at 2mg/kg/D. Other supportive care will be administered per institutional practice.

# Pre-Study Observations

**7.1. Visit I: Screening**

Patients will be informed by the investigator about the study at the screening visit; this will be recorded and documented appropriately. Written informed consent has to be obtained at the screening visit. No study related procedures will be performed before written consent has been obtained.

# Pre CTL Infusion Observations

* + 1. History and physical examination: A complete history and physical examination including weight, height, BSA.
		2. Hematology (must be within one week prior to starting therapy): WBC, differential, platelet count.
		3. Chemistry: Electrolytes, serum creatinine, BUN, total and direct bilirubin, SGPT(ALT), SGOT (AST), albumin, calcium, phosphorus, uric acid and magnesium, LDH.
		4. Performance Status: Karnofsky or Lansky (age appropriate)
		5. Baseline Chimerism Study: on CTL donor and recipient
		6. Urine and Plasma or Serum BK qRT-PCR
		7. HLA typing: HLA A and B by intermediate resolution; DRB1 by high resolution on donor and recipient
		8. Urine pregnancy test for females of child bearing age
		9. Characterization on Validations product of BKV-CTLs (Appendix 3) (Minimum 3 validations per site) (only on manufacturing validations)
		10. Characterization and Functional Assessment of the BKV CTL Clinical Grade Product (Appendix 4) (only on clinical product)
		11. Detection of BKV CTLs in the Blood of Patients Prior to Infusion of BKV CTLs (Appendix 5)
		12. HLA Antibodies (Appendix 12)
		13. All other laboratory monitoring according to the treating physician/standard of care

# Post BK CTLs Infusions Observations

* + 1. CBC with manual differential, platelet count weekly (±3 days) through week 12 post last CTL infusion
		2. GVHD: weekly assessment of stage and grade of both acute and chronic GVHD and as clinically indicated (Appendix IV)
		3. Urine and plasma or serum BK qRT-PCR for BK weekly (± 3 days) or more often if clinically needed, through week 12.
		4. Immune Studies: Quantitative immunoglobulins and quantitative CD3+ CD4+, CD8+, CD19+ and CD3-/CD56+ peripheral blood counts on days 60, 100, 180 and 365 (±10 days) post last BK CTL infusion
		5. Performance Status: Karnofsky or Lansky to be documented at Day +30, 100, 180 and 365 (±10 days) post last CTL infusion
		6. Correlative Biology studies will be measured on days +14 (±3 days) after first viral CTL infusion, and 60, 100 (±10 days) post last infusion of CTLs, (Appendix 6)
		7. Persistence of CTLS: Donor chimerism will be obtained on day 14 (±3 days) after the first dose of BK CTLs.

# Preparation of the BK CTLs

* 1. **Manufacture**
		1. Manufacturing of the BK CTLs preparations will be performed in the institutional stem cell processing laboratory. The manufacturing process and quality control will be performed according to validated procedures and documented in accordance with full GMP requirements.

The individual, donor-derived blood product (whole blood or leukapheresis product) will be incubated with PepTivator® Peptide Pools of BKV VP1 and BKV LT. After incubation, virus-specific cells will be enriched using the CliniMACS Cytokine Capture System (IFN- gamma). The entire preparation process will be performed using the fully automated CliniMACS Prodigy. If the total number of cells in the BK-specific T cells exceeds the number defined for the first dose of BK-CTLs, the remaining BK-CTLs will be cryopreserved. They may be given at a later time up to the defined total maximum dose, if necessary.

# Assessment

Products will be assessed for IFNg+ T cell content (CD4+/IFNg+ and CD8+/IFNg+) by flow cytometry using validated methods.

# Release criteria for final products

1. Within the T cell population (CD3+), viability of >70%, fresh or prior to cryopreservation

+

1. Among the CD4 and CD8 T cells, IFN
2. Negative gram stain.

cells are >10% of total is the target goal.

1. Additional required test: Endotoxin testing is performed on a sample of the final infusion product. The results of this test will not be available until after the product has been

infused. If the endotoxin values are >5 EU/kg of the recipient weight the PI must be notified

# Packaging, Labeling and Storage

* + - 1. Labeling

The BK-specific T cells bags will be labeled in accordance with FDA applicable regulatory requirements.

* + - 1. Storage

The BK-specific T cells are intended for direct administration after preparation. Shelf-life is 6 hours from the end of the processing.

In cases of donor timing issues or final product exceeding the first maximum dose, cells will be cryopreserved according to relevant institutional SOPs and thawed at the time of infusion. For cryopreservation, the cells are combined with equal volumes of a cryoprotectant containing 20% Dimethyl Sulfoxide (DMSO) in 5% Human Serum Albumin (HSA). After the addition of the 2X cryoprotectant to an equal volume of the cell suspension (1:1) the final concentration of DMSO will be 10%. The products then undergo automated controlled rate freezing with recording of the freezing curves and is stored in the vapor phase of liquid nitrogen in a monitored and alarmed freezer.

# STATISTICAL CONSIDERATIONS AND DATA ANALYSIS PLAN

* 1. **Accrual and Duration**

We plan to investigate 20 evaluable patients in cohort 1 and up to 10 evaluable patients in cohort 2 with an estimated duration of 3 years and with at least 6 months follow up on the last treated patient.

# 9.2. General Considerations

The statistical analyses in this study will be exploratory since the study is not powered to address any pre-defined statements but to generate valid hypotheses on safety/tolerability and efficacy issues. Thus, all resulting p-values and confidence intervals are to be interpreted in the exploratory sense, only. All analyses (safety and efficacy) will be performed for cohort 1 and cohort 2 separately.

Based on previous data, it is expected, that approximately 5% of patients will develop acute GVHD grade 3 and 4 probably or directly related to BK CTL infusion. For cohort 1, with 20 patients and 1 observed Grade 3/4 acute GVHD probably or directly related

to BK CTL infusions, the estimated Grade 3/4 GVHD rate will be 5% with 95% exact confidence interval (CI) [1%, 25%],which provides reasonable precision for the estimated GVHD rate. For the efficacy endpoint, we expect that approximately 25% of patients will obtain a complete response to BK-CTLs with undetectable viral load by qRT-PCR by week 12. With 20 patients, this implies to 5 responses and the 95% exact CI for the 25% response rate will be [9%, 49%], again providing reasonable precision for the estimated response rate. For cohort 2, the sample size is determined based on feasibility rather than statistical properties. With 10 patients and 1 observed Grade 3/4 acute GVHD, the estimated Grade 3/4 GVHD rate will be 10% with 95% exact CI [0.2%, 45%], and if no Grade 3/4 acute GVHD is observed then the estimated rate will be 0% with 95% exact CI [0%, 31%]. For the efficacy endpoint, with 10 patients, if the number of responses is 2 then the estimated response rate will be 20% with 95% exact CI [3%, 57%], and if the number of responses is 3 then the estimated response rate will be 30% with 95% exact CI [7%, 65%].

Data will be appropriately summarized and analyzed using tabulation and graphs for demographic and baseline characteristics, safety and efficacy observations and measurements. Standard descriptive summary statistics (i.e., n, arithmetic mean, standard deviation, median, lower/upper quartiles, and minimum/maximum values) will be calculated for continuous variables. Categorical data will be presented in frequency tables using counts and percentages.

The main analysis will be performed after completion of Week 12 after BK CTL infusion i.e., when all patients have either completed Week 12 after BK CTL infusion, are lost to follow-up or have died within this period. Additional analyses will be done on the 6-months post-transfer follow-up data and on the 1-year post-transfer follow-up data (end of follow-up), i.e., when all patients have completed the 6-months or 1-year period after BK CTL infusion, are lost to follow-up or have died within these periods.

Any deviations from the planned analyses will be described and justified in the final integrated study report. Statistical programming and analyses will be performed using the validated computer software package SAS® or other validated statistical software as required.

# Analyses of the Primary Endpoints

## Safety

The primary safety endpoint will be the incidence and severity of Grade III-IV acute GVHD within 8 weeks that is probably or directly related to BK-CTL infusion after the

last BK CTL infusion. The acute GVHD will be assessed and graded according to Appendix IV. The secondary safety endpoints will be Grade III/IV infusional toxicity, hematopoietic graft failure, and/or cytokine response syndrome probably or definitely related to BK-CTLs.

Frequency tabulations of the number and percentage of patients with acute GVHD by severity (i.e., the ‘crude incidence rates’) will be presented and displayed graphically together with the two-sided 95% confidence interval.

## Efficacy

* + - 1. The primary efficacy endpoint will be the percentage of patients with undetectable BK viral load, as measured by qPCR by Week 12 after first dose of BK- CTLs (maximum response).

Frequency tabulations of the number and percentage of patients with decrease in BK viral load, as measured by qPCR, will be presented and displayed graphically together with the two-sided 95% confidence intervals.

* + - 1. Definition of Response to BK-CTLs

Complete Response: BK-PCR in urine or plasma undetectable per lower limits of each institutional assay.

Partial Response: Decrease in viral load in urine or plasma by PCR of at least 1-log from baseline.

Stable Disease: Changes insufficient to qualify as a CR, PR or progression.

Progressive Disease: Increase in viral load in urine or plasma by PCR of at least 1-log from baseline.

# Analyses of the Secondary Endpoints

All inferential analyses for the secondary outcome variables will be interpreted in the exploratory sense, only.

Standard descriptive summary statistics (i.e., n, arithmetic mean, standard deviation, median, lower/upper quartiles, and minimum/maximum values) will be calculated for continuous variables. Categorical data will be presented in frequency tables using counts and percentages. Graphical presentation will be given by means of box and whisker plots and bar charts, as appropriate.

Time to occurrence of acute GVHD of any grade or to occurrence of chronic GVHD will be evaluated to assess incidence and severity of acute or chronic GVHD from day of BK CTL infusion. The first day of GVHD onset at a certain grade will be used to calculate a cumulative incidence curve for that GVHD grade, acute or chronic. Overall cumulative incidence curves will be computed along with the 95% confidence intervals until Week 12 after BK CTL infusion with death considered as a competing risk.

Survival distributions will be estimated using the Kaplan-Meier method. Binomial proportions will be estimated using the observed proportion. Incidence rates will also be estimated using the cumulative incidence function.

All adverse events data will be listed in the individual patient data listings, including all information documented on the adverse event form. Separate listings will be provided likewise for serious adverse events, adverse events in subjects who died, and for adverse events leading to discontinuation of the study.

# Safety Monitoring

A DSMB will review patient information and safety data quarterly or earlier as needed, and at 45 days or later after the third of three patients 12.00 years of age or older are infused with BK-CTLs and if necessary at 45 days after the sixth patients is infused with BK-CTLs (Section 9.3.4) with particular attention to Grade III-IV acute GVHD probably or directly related to BK-CTL infusion. These three patients have already been safely analyzed and will be included in the final 20 cohort. Patient safety will be assessed continuously throughout the study by monitoring incidence and severity of acute GVHD. Cohort 1 and 2 patients will be assessed separately for safety monitoring.

# Statistical Stopping Guidelines (Cohort 1)

* + 1. Acute GVHD

Acute GVHD grade III-IV will be monitored and incidence rates will be reviewed by the DSMB at least quarterly throughout the study.

The interim looks will be forwarded to the Sponsor and the DSMB. If rates significantly exceed pre-set statistical thresholds at the interim looks, further recruitment will be stopped and the Sponsor will decide about further study continuation after consultation with the DSMB. We expect that the probability of experiencing grade III-IV GVHD probably or related to BK-CTL infusion will be about 0.05 but will not accept the probability to be greater than 0.20.

The statistical stopping guidelines presented here are to serve as a trigger for initiating consultation with the DSMB for additional review. They are not intended as formal ‘stopping rules’ that would mandate automatic closure of study enrollment.

Grade III-IV Acute GVHD will be monitored continuously, after enrolling five patients until the end of the study. We expect the probability of Grade III/IV AGVHD to be 5%. The stopping rule will be triggered if there is significant evidence that the event rate exceeds 20%, that is, if the lower bound of the one-sided 95% CI exceeds 20%. If the number of patients with an acute GVHD grade >2 equals or exceeds the number in the tables below, then the study should be suspended pending further evaluation. For example, if 5 or more out of 10 subjects have grade >2 acute GVHD, the study will be suspended. Under this stopping rule, we would stop the study early with a probability of 0.3% if the true grade III-IV acute GVHD event rate is 10%, stop early with a probability of 8.7% if the true event rate is 20%, stop early with a probability of 71.2% if the true event rate is 40%, and stop early with a probability of 89.8% if the true event rate is 50%. These probabilities are calculated from a simulation study.

|  |  |
| --- | --- |
| Number of patients | Stop if grade >2acute GVHD >= |
| 5-7 | 4 |
| 8-10 | 5 |
| 11-14 | 6 |
| 15-17 | 7 |
| 18-20 | 8 |

Statistical stopping guidelines referring to incidence of Grade III-IV acute GVHD until Week 12 after BK CTL infusion have been defined to ensure patients’ safety throughout the study.

* + 1. Infusional Toxicity, Hematopoietic Graft Failure, and/or Cytokine Release Syndrome Probably or Directly Related to BK-CTL Infusion

For safety monitoring, infusional toxicity > grade 3 (NCI CTCAE v 4.0), hematopoietic graft failure and CRS >grade 3 97 will be monitored continuously, after enrolling five patients until the end of the study. The stopping rule will be triggered if there is significant evidence that the percent of patients with >grade 3 infusional toxicity or CRS exceeds 10%, that is, if the lower bound of the one-sided 95% CI exceeds 10%. If the number of patients with an infusional toxicity grade ≥ 3 equals or exceeds the number in the tables below, then the study should be suspended pending further evaluation. For example, if 4 or more out of 14 subjects have grade ≥ 3 infusional toxicity, the study will be suspended. Under this stopping rule, we would stop the study early with a probability of 0.5% if the true grade ≥ 3 infusional toxicity event rate is 5%, stop early with a probability of 7% if the true event rate is 10%, stop early with a probability of 63.2% if the true event rate is 25%, and stop early with a probability of 90.2% if the true event rate is 35%. These probabilities are calculated from a simulation study.

|  |  |
| --- | --- |
| Number of patients | Stop if grade ≥ 3infusional toxicity, hematopoietic graft failure or CRS >= |
| 5-8 | 3 |
| 9-14 | 4 |
| 15-21 | 5 |

* + 1. : Acute GVHD (Cohort 2)

Safety monitoring in Cohort 2 will follow the same principle as in Cohort 1. Grade III-IV Acute GVHD will be monitored continuously, after enrolling five patients until the end of the study. We expect the probability of Grade III/IV AGVHD to be 5%. The stopping rule will be triggered if there is significant evidence that the event rate exceeds 20%,

that is, if the lower bound of the one-sided 95% CI exceeds 20%. If the number of patients with an acute GVHD grade >2 equals or exceeds the number in the tables below, then the study should be suspended pending further evaluation. For example, if 4 or more out of 7 subjects have grade >2 acute GVHD, the study will be suspended. Under this stopping rule, we would stop the study early with a probability of 0.2% if the true grade III-IV acute GVHD event rate is 10%, stop early with a probability of 5.4% if the true event rate is 20%, stop early with a probability of 40% if the true event rate is 40%, and stop early with a probability of 64% if the true event rate is 50%. These probabilities are calculated from a simulation study.

|  |  |
| --- | --- |
| Number of patients | Stop if grade >2acute GVHD >= |
| 5-7 | 4 |
| 8-10 | 5 |

* + 1. : Infusional Toxicity, Hematopoietic Graft Failure and/or Cytokine Release Syndrome Probably or Directly Related to BKV-CTL Infusion (Cohort 2)

Infusional toxicity > grade 3 (NCI CTCAE v 5.0), hematopoietic graft failure and CRS

>grade 3 17 will be monitored continuously, after enrolling five patients until the end of the study. The stopping rule will be triggered if there is significant evidence that the percent of patients with >grade 3 infusional toxicity or CRS exceeds 10%, that is, if the lower bound of the one-sided 95% CI exceeds 10%. If the number of patients with an infusional toxicity grade ≥ 3 equals or exceeds the number in the tables below, then the study should be suspended pending further evaluation. For example, if 3 or more out of 8 subjects have grade ≥ 3 infusional toxicity, the study will be suspended. Under this stopping rule, we would stop the study early with a probability of 0.3% if the true grade ≥ 3 infusional toxicity event rate is 5%, stop early with a probability of 3% if the true event rate is 10%, stop early with a probability of 34% if the true event rate is 25%, and stop early with a probability of 65% if the true event rate is 35%. These probabilities are calculated from a simulation study.

|  |  |
| --- | --- |
| Number of patients | Stop if grade ≥ 3infusional toxicity, hematopoietic graft failure or CRS >= |
| 5-8 | 3 |
| 9-10 | 4 |

# Overall survival rate (OS)

Overall survival is defined as time from BK CTL infusion to death or last follow-up and will be assessed first at Day 1 and then throughout the study.

# Adverse Events: Definitions

The severity of adverse events (AEs) will be graded on a scale of 1 to 5 according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (The NCI Common Terminology Criteria for Adverse Events, Version 5.0 [NCI CTCAE]). The NCI CTCAE can be viewed on-line at the following NCI web site: [(http://ctep.cancer.gov/reporting/ctc.html).](http://ctep.cancer.gov/reporting/ctc.html%29)

# Definitions

* + 1. “Adverse event” means any sign, symptom, or clinically significant abnormal laboratory finding occurring during the study with the use of the investigational product. An adverse event should not be reported if a patient is entered on a study with a pre- existing condition unless the adverse event increases in severity or resolves and then returns while the subject is enlisted on the study. Assessment of adverse events will start on the first day of chemotherapy.
		2. An adverse event is considered "serious" if, in the view of the investigator/sponsor, it results in any of the following outcomes. Serious adverse event is now defined as any SAE possibly, probably or definitely related to multi-viral t-cell infusion causing any one of the following complications:
			1. Death,9.6.2.2 A life threatening adverse drug (cell therapy) experience,
			2. Inpatient hospitalization or prolongation of existing hospitalization,
			3. A persistent disability/incapacity,
			4. A congenital anomaly/birth defect, or
			5. Serious medical conditions defined as:
				1. Grade 3-5 infusion reaction according to the NCI CTCAE v5.0 possibly, probably, or definitely related to BK-CTL infusions within the first 24 hours after infusion
				2. Recipient seroconversion to any FDA-listed relevant communicable diseases within 6 months of BK-CTL infusion, which upon investigation, is determined to be caused or potentially caused by the BK-CTLs;
				3. Recipient bacteremia secondary to contaminated BK-CTLs
				4. Recipient develops any of the FDA listed relevant communicable diseases within 6 months of BK-CTL infusion which upon investigation is determined to be caused or potentially caused by the BK CTLs.
				5. Any grade 3-5 adverse event considered probably, or definitely related to BK- CTLs
		3. Dose limiting criteria defined as:
			1. Grade 3-5 infusion reaction, hematopoietic graft failures or CRS according to the NCI CTCAE v5.0 probably, or definitely related to BK-CTLs infusions
			2. Hematopoietic graft failure, if applicable, is defined as patients following allogeneic stem cell transplantation with a neutrophil count < 500/mm3 x 7 consecutive days with donor chimerism < 20% after 60 days post allogeneic stem cell transplantation.

Any grade 3-5 adverse event considered probably or definitely related to the BK CTLs infusion

* + - 1. Important medical events that may not result in death, be life- threatening, or require hospitalization may be considered a serious adverse experience when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of these outcomes.

# Reporting

* + 1. **Reporting of Serious Adverse Events to the Sponsor/PI, Dr. Mitchell Cairo, and two study Co-Chairs, Drs. Nancy Bunin and Julie Talano, and Institutional Review Board reporting**

All SAEs as defined in section 9.7, possibly, probably or definitely related to any of the BK-CTLs infusions will be reported to the:

Sponsor/PI (Mitchell Cairo, MD 914-594-2150 Mitchell\_Cairo@NYMC.edu), Study Co- Chair (jtalano@mcw.edu; buninn@email.chop.edu) and Clinical Research Nurse (Lauren Harrison, RN 617-285-7844/lauren\_harrison@nymc.edu) within 24 hours by email and a written report within seven working days after the occurrence of the incidence. The sub-site investigator shall report all internal adverse events that are determined to be serious and unanticipated to their local IRB according to institutional policy within 48 hours of the event, or notification of its occurrence. Submission of a written report by fax, hand delivery, or express mail delivery to the IRB office is acceptable. In filing the report, the investigator must make the preliminary determination whether revision(s) to the protocol and/or consent document(s) is/are necessary in coordination with the overall Co-Chairs. If a change is required, a modification must be submitted promptly to the IRB.

* + - 1. Adverse events with commercial agents that are “serious” as per the above definition, unexpected, and have an attribution of possible, probable or definite to a study drug, must also be reported to the FDA and Miltenyi, using a MedWatch form.

# Deaths unrelated to serious adverse events

Regardless of cause or whether a patient is on-study or off-study, all deaths must be reported to the Sponsor/PI and Study Co-Chairs within 48 hours. The Sponsor/PI and Study Co-Chairs will review the circumstances surrounding the patient’s death to confirm it does not constitute a serious adverse event, and the date and cause of death will be documented in the patient’s research chart.

# Investigator Reporting to the FDA

* + - * 1. Serious adverse events (SAEs) that are unlisted/unexpected, and either probably or directly related to BK CTLs, and that have not previously been reported in the Investigators Brochure, for this study should be reported promptly to the Food and Drug Administration (FDA) by telephone or by fax. Fatal or life threatening SAEs that meet the criteria for reporting to the FDA must be reported to the FDA within 7 calendar days after awareness of the event. All other SAEs that meet the criteria for reporting to the FDA must be reported to the FDA within 15 calendar days after awareness of the event. A clear description of the suspected reaction should be provided along with an assessment as to whether the event is drug or disease related.

# Participating sub-sites should NOT report SAEs to the FDA. The IND holder, Mitchell S. Cairo will be responsible for reporting to FDA.

* + - 1. If the integrity of the BK CTL product is compromised at receipt (or any time after receipt), it is reported immediately to Dr. Mitchell Cairo, IND holder. Examples include: a broken unit, or a contaminated product. The occurrence is then investigated per Quality Improvement process and if the events are believed to be related to the manufacturing of the distributed multiviral t-cell product, this will be reported to the FDA within 14 days.

# Off Study Criteria (Any one or more of the following)

* 1. Removal from study secondary to BK CTL grade III or IV infusion toxicity
	2. Progression of BK infection requiring new systemic anti-BK therapy
	3. Patient/Parent refusal to continue
	4. Physician investigator determines it is not the best interest of the patient to continue therapy.
	5. One year from Day 0 of the last BK-CTL infusion.
	6. Lost to follow-up

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# Appendix I: Evaluation of Donors for Apheresis

In addition to donor evaluations below- donor must first be assessed to have a T-cell response at least to the BK MACS® GMP PepTivator antigen(s) causing the therapy- refractory infection. The donor serology will also be assessed for BK.

1. **Principle:** Related allogeneic donors are required to meet transmissible infectious disease screening and testing requirements. This requires evaluation of risk factors, review of medical records, physical examination, and testing for relevant communicable disease agents and diseases (RCDADs) in accordance with the Code of Federal (CFR) Regulations**:** [CFR: Tissue Donor Eligibility](http://www.ecfr.gov/cgi-bin/retrieveECFR?gp=1&SID=fcc5180f537827131e00724c65272823&h=L&mc=true&n=sp21.8.1271.c&r=SUBPART&ty=HTML)
2. **Purpose:** The donor is evaluated to protect the safety of the recipient.

# Procedure:

* 1. **Determination of the allogeneic donor eligibility**
		1. Responsibility of the transplant physician and is communicated to the collection and processing enter staff.
		2. Eligible Donors:
			+ Screening shows that the donor is free from risk factors for, and clinical evidence of, infection due to RCDADs, and is free from communicable disease risks associated with xenotransplantation; and
			+ Test results for RCDADs are negative or nonreactive, except as provided in § 1271.80(d)(1): active on a non-treponemal screening test for syphilis and negative on a specific treponemal confirmatory test;
		3. Ineligible Donors:
			+ Require documentation of the rationale for his/her selection by the transplant physician, urgent medical need and documentation of informed consent of the donor and the recipient.

# Donor Health History Review

* + 1. Rationale

The purpose of the health history review is to assess the donor’s current state of health and risk RCDADs as defined by the Good Tissue Practices (GTPs) and listed/specified in 21 CFR Part 1271. These are diseases or disease agents

identified by the FDA as having the potential to cause significant pathogenicity to recipients of human cells, tissues, and cellular and tissue-based products (HCT/Ps). RCDADs are determined by assessing:

* Risk of transmission to the recipient.
* Severity of effect on the recipient if transmitted.
* Availability of appropriate screening measures or tests to identify the potential donor’s risk of exposure to and/or possible infection with the disease.

RCDADs include West Nile Virus (WNV), HIV-1/2, hepatitis B, hepatitis C, vaccinia virus infection, HTLV I/II, Chagas, Creutzfeldt-Jakob disease (CJD), variant CJD, sepsis and syphilis

3.2.2 Donor Questionnaire

The clinical program will use a donor questionnaire and guidance document that is based on the National Marrow Donor Program’s Donor Heath History Screening Questionnaire.

3.2.3 Evaluation of response to Donor Questionnaire

Responses will be assessed for risk of RCDADs as defined by the Good Tissue Practices (GTPs) and listed/specified in 21 CFR Part 1271.50.

* + - 1. Risk of RCDADs is identified Donor is determined ineligible.
			2. Other atypical response identified

Atypical responses to the screening questions must be evaluated on a case-by-case basis to determine donor eligibility.

* 1. **Infectious disease (ID) evaluation** within 30 days prior to collection will include:
* HIV Ab (NAT testing)
* HTLV I/II Ab
* HBsAg
* Anti-HBcV
* Anti-HCV (NAT testing)
* Anti-BK, BK- urine
* EBV serology
* Serologic test for syphilis West Nile Virus.
* Trypanosoma cruzi (Chagas’ Disease)
	+ 1. Incomplete or > 30 day old ID testing

Donor is determined ineligible.

# Donor Confidentiality:

Any findings determined by the transplant physician to require follow up will be discussed with the donor or donor guardian(s). Findings will remain confidential.

# REFERENCES:

1. Foundation for the Accreditation of Cellular Therapy, Standards for Hematopoietic Progenitor Cell Collection, Processing and Transplantation, Sixth Edition 2015.
2. Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products, U.S. Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research, August 2007

# B. DONOR EVALUATION AND SCREENING FOR SUITABILITY

1. **Principle:** Standards mandate criteria for allogeneic donor selection, evaluation, and management by trained medical personnel for both safety of the donor and recipient.
2. **Purpose:** The related donor is evaluated to protect the safety of the donor and recipient.

# Procedure

* 1. **Suitability –** Applies to allogeneic donors.
		1. Donors will be evaluated to determine if safe to proceed with the collection procedure. The evaluation includes the following:

. **3.1.1.1** Medical history and physical examination.

* + - 1. Laboratory evaluation including CBC, chemistry panel, Mg, urinalysis, ABO and Rh.
			2. The donor is evaluated for potential risks of the following collection procedures:
				* Possible need for central venous access.

\*Donors for PSC will be evaluated by Apheresis. Donors must have adequate catheter or venous access for procedure

* + - 1. If the donor has a condition for which he/she may be at risk during the procedure (*e.g.* asthma, cardiac problems), he/she will be evaluated by an appropriate physician and/or anesthesia prior to initiation of conditioning.
			2. Pregnancy assessment all female donors with childbearing potential within seven (7) days prior to collection.
		1. The use of a donor who does not meet Clinical Program donor safety criteria will be documented with the rationale for his/her selection by the transplant physician.
		2. Any abnormal finding of the prospective donor is documented in the in the donor record with recommendations made for follow-up care.
		3. Issues of donor health that pertain to the safety of the collection procedure are communicated in writing to the Collection Facility staff.

# Donor Confidentiality:

Any findings determined by the transplant physician to require follow up will be discussed with the donor or donor guardian(s). Findings will remain confidential.

# REFERENCES:

1. Foundation for the Accreditation of Cellular Therapy, Standards for Hematopoietic Progenitor Cell Collection, Processing and Transplantation, Sixth Edition 2015.
2. Confer, DL. Stroncek, DF. Bone Marrow and Peripheral Blood Stem Cell Donors. In: Thomas Ed, Blume KG, Forman SJ (eds). Hematopoietic Cell Transplantation 2nd Ed. Malden, MA: Blackwell Science, Inc: 1999:421-430.

# Appendix 2: Viral CTL Manufacturing

* 1. **vCTL MANUFACTURING USING THE CliniMACS PRODIGY**
	2. **Principle**
	3. Viral infection or reactivation following transplant (stem cell or solid organ) is a significant cause of morbidity and mortality. Pharmacologic intervention can be helpful, but has associated toxicity and many patients are refractory.
	4. The use of Cytotoxic T Lymphocytes (CTLs) against these viruses have been used to treat these patients in clinical trials and have shown promise. However, traditional methods of generating these cells involve weeks of culturing and are very labor intensive.
	5. The CliniMACS Cytokine Capture System (CCS) developed by Miltenyi is a method to isolate virus specific T cells by stimulating them with virus specific peptides. They will then secret interferon (IFN ), which will be captured on the cell surface using a catch reagent that is essentially a bivalent antibody against both CD45 and IFN . IFN -coated cells are then labelled with another anti- IFN antibody conjugated with paramagnetic beads and enriched. The CliniMACS prodigy device is a multi-purpose cell processor that will render this procedure largely automated.
	6. This method can be used to generate viral specific CTLs (vCTLs) against a single, or multiple viruses, depending on the composition of the stimulating viral peptides. Viral specificity can be assessed by culturing a small portion of the final product with mitotically inactivated feeder cells and expanded for 2 weeks, at the end of which they can be re-stimulated with the same viral peptides individually to assess IFN response.

# Purpose

The purpose of this procedure is to describe the steps to follow in the manufacturing of vCTLs and the subsequent culture expansion for re-testing.

# Specimen

* 1. T cells, Apheresis
	2. T cells, Whole Blood

# Supplies Source

* 1. Bag access Alaris
	2. Syringes BD
	3. Needles BD
	4. Sterile Fields Medchoice
	5. Alcohol wipes ITW Textwipe
	6. Human serum albumin, 25% Pharmacy
	7. Human serum albumin, 5% Pharmacy
	8. CliniMACS PBS/EDTA buffer (3L) Miltenyi
	9. CliniMACS PBS/EDTA buffer (1L) Miltenyi
	10. Lymphocyte separation medium MP Biomedicals, LLC
	11. 50 ml polypropylene centrifuge tubes Sarstedt
	12. 50 ml polystyrene centrifuge tubes Corning
	13. TexMACS GMP medium (2L) Miltenyi
	14. MACS GMP Peptivator BKV VP1 and BKV LT (60nmol) Miltenyi
	15. CliniMACS cytokine capture system Miltenyi Containing: 7.5 mL CliniMACS IFN Catchmatrix Reagent

7.5 mL CliniMACS IFN Enrichment Reagent

* 1. 0.9% Sodium Chloride as elution buffer Baxter
	2. Prodigy TS500 tubing set Miltenyi
	3. Research Peptivator BK pp65 (6nmol) Miltenyi
	4. IFN-gamma staining Miltenyi
	5. Rapid Cytokine Inspector kit Miltenyi
	6. Interleukin 2 (25 g) Miltenyi
	7. Pennicillin-Streptomycin Gibco
	8. sterile water (250ml) Baxter
	9. Pipets, individually wrapped Fisher
	10. Plasma transfer sets with two couplers Fenwal
	11. Transfer bags (1000ml, 600ml, 3000ml) Fenwal
	12. Trypan Blue 0.4% in PBS Invitrogen
	13. Pipet tips Fischer Scientific
	14. Gloves SPD
	15. 15ml centrifuge tubes Corning
	16. Tissue culture plates (24, 48well) Corning
	17. Tissue culture flasks (vent cap) Corning
	18. Cryotube vials (2ml) Nunc

# Equipment

* 1. CliniMACS Prodigy device
	2. Biological Safety Cabinet
	3. Refrigerated centrifuge
	4. COBE 2991
	5. Pipet-aid
	6. Pipettors 100-1000µl, 20-200µl, 2-20µl
	7. CO2 incubators
	8. Sebra heat sealer
	9. Terumo sterile tubing welder
	10. Microscope
	11. ACT II diff Hematology Analyzer
	12. Hemostats
	13. FACSCalibur Flow cytometer
	14. Remote monitoring camera
	15. laptop
	16. Hemacytometer

# Forms/Requisitions/Labels/Log Book

* 1. Physician's order
	2. Processing record review form
	3. Acceptance of a stem cell product form
	4. Blood Bank acceptance of a stem cell product (if applicable)
	5. Stem Cell Product release form (for products infused fresh)
	6. Infusion form (for products infused fresh)
	7. vCTL manufacturing flowsheets
	8. Microbiology requisitions
	9. Blood Bank requisitions
	10. Hematology requisitions
	11. Endotoxin requisition form
	12. LABS requisition form
	13. Patient's identification labels
	14. Intermediate labels
	15. Certificate of analysis

# Procedure

**vCTL manfacturing**

* 1. When the product is received in the laboratory, assign to it the unique identification and enter it in the log book. If the identification number had been assigned before the collection, enter the date the product was received.
	2. The cell product should be processed on the day of collection, if at all possible. When the product has to be stored overnight, store it per institutional cryopreseration SOP.
	3. Insert a bag access device into one of the bag ports. Using a syringe, remove a small volume of product (5.0 ml) for sterility testing, for nucleated cell count, viability count, for CBC and differential.
		1. The cell concentration should not exceed 2.0 x 108/ml during storage. Dilute the product in autologous plasma or in 5% HSA if needed.
	4. Prepare processing buffer by making 0.5% (w/v) HSA into PBS/EDTA buffer. So for a 3L bag, add 60ml of 25% HSA. For a 1L bag, add 20ml.
	5. Aliquot 1x109 total nucleated cells for the manufacturing process on the Prodigy.
		1. If the product is a whole blood product, perform a RBC reduction using institutional RBC depletion SOPs. Since the expected recovery is at least 23%, a minimum of 4.44 x109 TNC is needed.
		2. An apheresis product even with a high Hct generally does not require RBC reduction because the small volume needed to achieve 1x109 cells.
		3. Final 1x109 cells are diluted and/or resuspended in 75ml of processing buffer before loading onto Prodigy.
	6. Turn on the Prodigy and choose **CCS-IFN enrichment** under the **Process** tab. Enter information as prompted using either 1) bar code scanner attached to the machine, or 2) manually using the onscreen keyboard brought out by touching **Edit** to the right of the screen.
	7. Install the TS500 tubing set on the Prodigy following instructions on the computer screen. While every step is clearly illustrated on the computer screen and in the overall picture below, the following areas warrant special attention:
		1. Connections near valves 9, 10 and 12, and pre-column tubing placements.
		2. Tubing flow around the pump is opposite of those on the CliniMACS Plus.
		3. When installing the Heat Exchange Cartridge (HEC) and Chamber into the CentriCult Unit (CCU), make sure 1) the top edge of HEC clicks into place,

2) the three tubings exiting the CCU fit comfortably thru the three slots, 3) the CCU also closes with a click.

* + 1. The waste bag is to be left on the bench due to expected large fill volume.
		2. Tubing set must pass both the upper and lower part of integrity test.
	1. After tubing set installation, the following steps are performed in order:
		1. Connect TexMACS media, 3L processing buffer and elution buffer (0.9% NaCl).
		2. Priming, which takes ~30min
		3. 200ml of elution buffer will have been transferred to the reservoir bag at this point. Supplement with 2.5% HSA by adding 22ml of 25% HSA.



* 1. Add 1x109 cells to the application bag. Resuspend the cells with PBS/EDTA/0.5%HSA to a final volume of 75ml. Sterile dock with the application bag to transfer the cells. Seal off and remove the QC pouch attached to the application bag as it is of no use in this procedure.
	2. Add reconstituted antigens(s)
		1. Dissolve lyophilized Peptivator powder with 8ml of sterile water by directly injection into the vial. Mix to dissolve and minimize bubble formation.
		2. Up to 5 different Peptivators can be combined into one pool. In this case, serially transfer the ~8ml volume from one vial to another until all are dissolved in one pool of ~8ml.
	3. Connect CCS Catchmatrix and Enrichment reagents.
	4. The processing will take about 12 hours, and is fully automated. Processing end time can be specified to coincide within working hours, which will result in a delayed start controlled by the computer. The entire process must not exceed 36 hours.
		1. A QC sample needs to be taken ~70 min before the end of processing. Planning should ensure this time point also happens within working hours.
	5. ~70min before end of processing, seal off the QC bag (antigen stimulated, but not yet IFN enriched) and keep at 4C. At the end of processing, collect the Target cells (TC) bag and non-target cell (NTC) bag, and analyze cell number and composition along with the saved QC.
		1. The TC fraction will be ~7-8ml in volume and may contain as few as 105- 106 cells. Draw 1ml for the following uses: 0.5ml for flow and 0.5ml for expansion. Measure the remaining volume, and dilute with 0.9% Sodium Chloride with HSA (50 ml of 25% HSA in a 1000 ml bag of 0.9% Sodium Chloride) to a final volume of 33ml.
		2. The QC bag contains a sample of 100ml Original fraction (ORI) of the pre- enriched cells. Perform cell count and flow analysis.
		3. Also perform cell count and analysis on the NTC. The volume of NTC can be determined by either weighing or measuring with a syringe. Save most

of the NTC to use as feeder cells for expansion of the IFN below)

+

cells. (See

* 1. For the 0.5ml TC saved for flow, perform cell count and split the rest of the cells 90%-10%, and label them as TC1 and TC2, respectively. Stain for flow as below:
		1. Add 0.5ml pre-chilled PBS/EDTA/0.5% HSA and centrifuge at 2700rpm x 5min.
		2. Make a master mix of (90 l cold PBS/EDTA/0.5% HSA + 10 l

IFN (PE) antibody + 10 l T Cell Detection Cocktail from the Rapid Cytokine Inspector (RCI) kit) x 3. Add the 110 l to three cell samples, (TC1 (90%), NTC, and ORI) and resuspend. For TC2 (10%), add CD45- FITC, CD3-PE antibodies. Incubate at 4C x 10min.

* + 1. Add 1ml of RBC lysis solution to all 4 and incubate 10min at room temp.
		2. Centrifuge at 2700rpm x 5min. Remove supernatant carefully, and resuspend cells in 0.5ml PBS/EDTA/0.5% HSA. Add 5 l 7AAD (0.05mg/ml) to a final concentration of ~0.5 g/ml and perform flow analysis following institutional SOPs.
	1. For the ORI and NTC fractions, there are sufficient cells to perform flow as normal samples. Pellet 1-2x106 cells and stain with TC together starting from 7.15.2.
		1. With total cell number only in the tens of thousands for the TC sample, acquire as many events as possible. For ORI and NTC samples, acquire 250,000-500,000 events.
		2. Use same gating strategy as described in Donor PreScreening for Virus Specific Cytotoxic T Lymphocytes.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | VioBlue | FITC | PE | PerCP | APC |
| RCIcocktail | CD3 | CD8 | -- | CD14/CD20 | CD4 |
| Separate add | -- | -- | IFN | 7AAD (just prior) | -- |
| Analysis | ND for Calibur | CD8 | IFN | Exclusion gate | CD4 |

* + 1. The TC2 tube is for obtaining the viability of CD3+ cells.
	1. The performance characteristic of the procedure is assessed by the following:
		1. Recovery (CD4 or CD8) = # IFN cells (TC) **/** # IFN cells (ORI)

* + 1. Corrected Recovery (CD4 or CD8) = # IFN cells (TC)

.

# IFN cells (TC) **+** # IFN cells

(NTC)

* + 1. Enrichment factor = % IFN cells (TC) % IFN cells (TC)

(CD4 or CD8) % IFN cells (ORI) % IFN cells (ORI)

* 1. Remove 3 ml from the 33ml product above to be sent to Microbiology (Gram stain), APPTEC for Endotoxin testing and LABS for sterility testing. The final product cannot be released until the gram stain result is available. The requisitions to Microbiology must be sent “stat”.

# The final product is held in the blood bank refrigerator (2°- 6°C) “in quarantine” until the test results are available.

The release criteria for products are as follows:

* The gram stain is negative.
* The cell viability is ≥70% within the T cell population, fresh or prior to cryopreservation.
* Among the CD4 or CD8 T cells, IFN cells are >10% of total is the goal.

+

The results obtained will be documented on the Certificate of analysis for the product. If the release criteria are not met, notify the Principal Investigator (PI) or, in his or her absence, one of the Co-investigators (CI) for the clinical protocol.

This notification is documented on the flowsheet for the procedure (Procedure outcome section).

When the tests results are available, the certificate of analysis is reviewed and signed by the Laboratory Director or her designee, or by institutional SOPs.

Deliver the product, accompanied by the required forms, to the patient unit for infusion following institutional infusion SOPs.

* 1. Download the Prodigy performance and reagent/supplies data by inserting a USB drive to the right side of the touch screen. Select **Filed Data** tab and highlight the data to be saved. Select **Save**. After file transfer, select **OK** to return to home screen and remove the USB stick. Shut down the machine by going to the **Settings** tab, then **tools**, then **shut down**.

**Cell expansion**

* 1. Arrange with blood bank to use their irradiator to inactivate the NTC cell fraction. These will be used as feeder cells at a ratio of 100:1 to TC. Aliquot 3x as many cells as needed and irradiate at 25Gy.
	2. Mix 100:1 with TC and culture at a starting concentration of 5x106 (total) cells/ml TexMACS at a density of 5x10e6 cells/cm2, supplemented to a final concentration of 100 IU/ml of IL2. Depending on the volume, use 48 or 24 well plates, with a surface area of ~1cm2/well or ~2cm2/well, respectively. Prepare a control well with NTC only at the same cell number. Culture in a 37C, 5% CO2 incubator.
		1. For example, 1x105 TC is mixed with 1x107 NTC, for a total of 1x107 cells. These will be put in 2ml final volume and a 24well plate.
		2. For example, 5x104 TC is mixed with 5x106 NTC, for a total of 5x106 cells. These will be put in 1ml final volume and a 48well plate.
	3. To make IL2, first prepare 1% HSA by diluting 5% HSA into sterile water. Resuspend lyophylized IL2 in 1% HSA to a final concentration of 10,000 IU/ml.
		1. The volume is dispersed in 1ml stocks and 50 l working stocks. The working stock is at 100x final concentration and is for single-use. 1ml stocks are used to make more working stocks.
		2. Label the outside container housing the aliquots with reagent name (10,000IU/ml IL2), storage temperature, lot number, date aliquoted, and expiration date. Label each aliquot container with the reagent name, volume (50 l or other volumes), lot number, expiration date, and hazard pictogram.
	4. Exchange half of the medium (without disturbing the cells) every 2-3 days with fresh medium containing fresh IL2. Split cells when appropriate (e.g., when

proliferating cells start to change media color). Since the only cells that will proliferate are the non-irradiated TCs, and they start at a very low number, first split may happen after 1 week of culturing and happens every 1-2 days after that.

* 1. Around day 10-14 of culture, most NTCs are expected to have died of apoptosis and only the proliferated TCs remain. A good expansion will see the TC expand

>100 fold in number. Test their viral responses by using individual viral antigens to re-stimulate following Donor PreScreening for Virus Specific Cytotoxic T Lymphocytes.

# Expected Results

* 1. Manufacturing on the Prodigy is expected to significantly enrich for IFN positive CD4/CD8 cells to a level of >10%.

.

# Quality Control Tests

* 1. Nucleated cell counts and viability counts are performed on the initial product, at different points during the procedure as indicated in the SOP and on the final product.
	2. Sterility testing is performed on the initial product and on the final product.
	3. Endotoxin testing is performed on the final product.
	4. CD4/IFN and CD8/IFN determination by Flow Cytometry is performed on the final product.

# References

* 1. CliniMACS Prodigy CCS System User Manual. Miltenyi Biotec.

# Donor PreScreening for Virus Specific Cytotoxic T Lymphocytes

1. **Principle**

In order to manufacture virus specific cytotoxic T lymphocytes (vCTLs), donor cells need to be prescreened to determine if the T cells can be stimulated with corresponding virus peptides. Positive serology for certain viruses is not a guarantee that the T cells will respond to the peptides used. Mononuclear cell preparation will be stimulated with either medium (negative control), virus specific peptide, or a combination of Phorbol 12- myristate 13-acetate (PMA) /ionomycine (positive control). The read-out is intracellular IFN-gamma measured by cell surface capture using a catch reagent, followed by flow cytometric analysis.

# Purpose

The purpose of this procedure is to describe the steps to follow while prescreening T cells from donors to determine their suitability for manufacturing vCTLs.

# Specimen

* 1. Collect 30 ml in EDTA Tubes on donor

# Supplies and reagents Source

* 1. PepTivator® BK pp65 (6nmol) Miltenyi Biotec
	2. TexMACS medium Miltenyi Biotec
	3. Rapid Cytokine Inspector kit Miltenyi Biotec
	4. IFN secretion assay detection kit Miltenyi Biotec
	5. Phorbol 12-myristate 13-acetate (PMA) Sigma
	6. Ionomycin calcium salt Sigma
	7. Sterile Fields Medchoice
	8. 15 ml centrifuge tubes Corning
	9. 50 ml centrifuge tubes Corning
	10. Tissue culture plate (24-well) Corning
	11. 12 x 75 mm test tubes BD Falcon
	12. Gloves SPD
	13. Pipets (5, 10, 25ml), individually wrapped Fisher Scientific
	14. Pipettor tips 1-100 µl, 101-1000 µl Fisher
	15. Sterile water Fisher
	16. Microcentrifuge tubes Fisher
	17. T25 tissue culture flask Nunc
	18. Dimethyl sulfoxide (DMSO) Origen
	19. Syringe (1cc) BD
	20. Needles (18 gauge) BD
	21. Alcohol wipes ITW Textwipe
	22. Lymphocyte separation medium MP Biomedicals, LLC

# Equipment

* 1. Biological Safety Cabinet
	2. Refrigerated centrifuge
	3. Pipet-aid
	4. Pipettors 100-1000µl, 20-200µl, 2-20µl, 1-10µl
	5. Microscope
	6. ACT II diff Hematology Analyzer
	7. Table top microcentrifuge
	8. CO2 incubator

# Forms/Requisitions/Labels/Log Book

* 1. Physician's order
	2. Acceptance of a stem cell product form
	3. Donor prescreening for vCTL flowsheet
	4. Blood Bank requisitions
	5. Virology requisitions
	6. Patient's identification labels
	7. Intermediate labels

# Procedure

* 1. Reconstitute the 6 nmol viral peptides (BK) with 200 L sterile water by directly injecting through the rubber stopper using a 1ml syringe. Vortex to mix. Make

20 L aliquots and store in ultra low freezer. Each aliquot is labelled as 0.6nmol BK. Label the outside container housing the aliquots with reagent name, volume, storage temperature, lot number, date aliquoted, and expiration date. Label each aliquot container with the reagent name, lot number, expiration date, and hazard pictogram.

* 1. The positive control used is 20ng/ml PMA and 1μg/ml ionomycin.
		1. Resuspend 1mg of PMA in 1ml DMSO (1mg/ml). Vortex to mix. Make

50 l aliquots and store at -20C. Make 1mg/ml ionomycin in DMSO the same way. Label the outside container housing the aliquots with reagent name, volume, storage temperature, lot number, date aliquoted, and expiration date. Label each aliquot container with the reagent name, lot number, expiration date, and hazard pictogram.

* + 1. To make the PMA+ionomycin needed for step 7.6, thaw an aliquot of the two reagents: Dilute 5 l of 1mg/ml ionomycin (in DMSO) with 85 l TexMACS medium. Vortex to mix. Dilute 10 l 1mg/ml PMA (in DMSO) into 990 l TexMACS medium (now 10μg/ml). Vortex to mix. Take 10 l of 10μg/ml PMA and add it to the 90 l of TexMACS containing ionomycin. Thus, this 100 l TexMACS contains 1μg/ml PMA and 50μg/ml of ionomycin.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Conc | Ddilution | Conc | Dilution | Conc |
| PMA | 1mg/ml | 1:100 (10 into990) | 10*μ* g/ml | 1:10 (10 into5+85) | 1*μ*g/ml |
| Ionomycin | 1mg/ml |  |  | 1:20 (5 into85+10) | 50*μ* g/ml |

After adding to the cells at 20 l into ~1ml (~1:50), final concentrations will be 20ng/ml PMA and 1μg/ml ionomycin.

* + 1. All aliquots used in steps 7.2 are for single use.
	1. To prepare freshly collected (room temperature or higher) cells for testing for this protocol, prepare a PBMC sample using Ficoll gradient by centrifuging at 1500 rpm x 16.5 min. Use at least 1-2x108 total nucleated cells. For apheresis product, this usually means ~1ml of volume, which can be diluted to 5ml using TexMACS medium and overlaid over 5ml of Ficoll in a 15ml tube. For whole blood or marrow, this usually means more volume, which can be directly overlaid on Ficoll in a 15 or 50ml tube.
	2. Cells that have been refrigerated/frozen:
		1. If using frozen cells, start the procedure a day before. Thaw a QC vial and resuspend the cells in 10ml TexMACS medium in a 15ml tube. Centrifuge at 1200 rpm x 10min to wash away the DMSO. Resuspend the pellet in a T25 tissue culture flask in TexMACS at a concentration < 1x107/ml, and let

the cells recover in a 37C incubator overnight. Non-viable cells and debris are expected.

* + - 1. Next day, mix the cells well and if possible, carefully pipette out the DNA aggregate or visible debris while minimizing cell loss. Perform a cell count and viability using trypan blue. Add fresh TexMACS and perform Ficoll as described in step 7.3.
		1. If using fresh cells that arrived in chilled state during transport/storage, culture them at 37C in TexMACS at < 1x107/ml for at least 1 hour and then perform Ficoll as described in step 7.3. (Example: an NDMP product arriving the night before processing and stored at 4C.)
	1. Collect the PBMC layer and transfer into a new 50ml tube. Add more TexMACS to 45ml and centrifuge at 1200 rpm x 16.5min to wash away Ficoll. Aspirate supernatant and resuspend cells in TexMACS to a final volume of 2ml. Perform a cell count. (With 2x108 starting cells and a hypothetical 30% recovery, this gives 3x107/ml x 2ml = 6x107 cells.) Adjust to a final live cell concentration of

~1x107/ml. (Take into account trypan blue viability if using thawed cells).

* 1. Add 1ml (~1x107 cells) to separate wells in a 24-well plate, with at least one empty well separating those with cells. Use 20 l TexMACS as negative control,

20 l of the peptide aliquot for each virus to be tested, and 20 L of the PMA+ionomycin mix (see step 7.2) as positive control. Always process the cells in such an order to minimize carry-over contamination. Mix carefully and place in 37C incubator with 5% CO2 for 4 hours.

* + 1. If cell numbers are not adequate, sacrifice positive control volume first, before reducing volume in other wells. A minimum of 0.5ml is needed to cover the well surface with adequate mixing. Reduce the 20 l media/peptide/PMA+ionomycin proportionally if <1ml of cells are used.
		2. This step and later ones, if needed, can be performed on an open bench.
	1. **COLD**. After 4 hours, mix the cells well and transfer 0.1ml (~1x106 cells) of each of the treatment into an microfuge tube. Add 0.4ml of cold TexMACS medium. Quick spin 1 min in the microcentrifuge to pellet the cells. Pipette out the media carefully and completely, careful not to disturb the pellet. Make a master mix of (90 L cold TexMACS + 10 L IFN catch reagent) x n, where n=number of samples. Add 100 l each to the cell pellet and resuspend. Incubate at 4C x 5min.
	2. **WARM**. After 5min, add 1ml of 37C TexMACS to each tube. Incubate upright in a 37C incubator for 45min, inverting the tubes every 5-10 minutes to prevent the cells from settling.
	3. **COLD.** After 45min, quick spin 1min in the microcentrifuge to pellet the cells. Remove supernatant carefully with a pipettor, be careful with the small pellet of

~1x106 cells. Resuspend in 1ml of cold TexMACS and pellet in microcentrifuge again. Remove supernatant carefully with a pipettor. Make a master mix of (90 l cold TexMACS + 10 l IFN (PE) antibody + 10 l CD4/CD8 T Cell detection cocktail) x n. Add 110 l each to the cell pellet and resuspend.

Incubate at 4C x 10min.

* 1. **COLD.** Wash cells by adding 1ml of cold TexMACS and quick spin 1min in the microcentrifuge to pellet the cells. Remove supernatant carefully with a pipettor, and resuspend cells in 0.5ml PBS. Add 5 l 7AAD (0.05mg/ml) to a final concentration of ~0.5 g/ml and perform flow analysis. Characterization of Cells by Flow Cytometry. Acquire 300,000-500,000 events.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | VioBlue | FITC | PE | PerCP | APC |
| RCIcocktail | CD3 | CD8 | -- | CD14/CD20 | CD4 |
| Separate add | -- | -- | IFN | 7AAD (just prior) | -- |
| Analysis | ND for Calibur | CD8 | IFN | Exclusion gate | CD4 |

RCI = Rapid Cytokine Inspection kit, providing the CD4/CD8 T Cell detection cocktail

* 1. Data analysis goes through the following gates:
		1. On FSC-SSC dot plot, gate on the lymphocytes (low FSC and low SSC).
		2. In the lymphocyte gate, display PerCP-SSC dot plot and exclude positive cells in the PerCP channel (CD14, CD20, 7AAD+ cells).
		3. In the remaining cells, display CD4/IFN and CD8/IFN , and gate on double positive cells.

# Expected Results

* 1. Unstimulated cells are expected to give little or no IFN + cells.

Stimulated cells are expected to give a small population of CD4/IFN and/or CD8/IFN double positive cells. One example provided by Miltenyi shows 0.058% CD8/IFN double positive cells. In the same example, CD4/IFN double positive cells are even fewer, at just 3 out of 614,897 cells, which will be below our cytometer’s limit of detection.

Cells stimulated with PMA/ionomycin should show marked increase in

percentage of IFN cells.

+

* 1. Generally speaking, the following criteria are considered when deciding if a donor is suitable for vCTL manufacturing:
* % IFNγ+ cells > 0.01 %
* At least 10 IFNγ+ events from 100,000 total events
* Twice the IFNγ+ events than the negative control

# Quality Control Tests

* 1. Nucleated cell counts and viability counts are performed on the initial product, at different points during the procedure as indicated in the SOP.
	2. CD4/IFN and CD8/IFN , determination by Flow Cytometry is performed on the post-stimulation product.

# References

* 1. Rapid Cytokine Inspector kit. Miltenyi Biotec.
	2. IFN secretion assay detection kit. Miltenyi Biotec
	3. CTS\_CCS staining strategy, prepared by Dr. Rebecca McHugh, Miltenyi Biotec.

# APPENDIX 3: Validation Products

1. **Objective:**

To validate and characterize the final vCTL product (IFG+ cells from CCS product) (only manufactured validations)

# Methods for Preparation of Cells

* 1. Each new center will perform 3 validations of either combination BKV, CMV, ADV or EBV CTLs.
	2. The 5-6 million cells of pre-stimulated donor PBMC, QC samples from Prodigy and the final validation products will be needed for the following studies:
		1. single cell RNAseq analysis- 1x10^5 cells of pre-stimulated PBMC, QC sample and target cells
		2. Nanostring Immunoprofiling: 5x10^5 to 1x10^6 cells from pre-stimulated PBMC, QC sample and target cells
		3. Mass Cytometry by Time of Flight (CyTOF): 1-2 x10^6 cells of pre- stimulated PBMC, QC sample and target cells
		4. T-cell Repertoire: 2x10^6 cells of pre-stimulated PBMC and QC sample and 3x10^5 target CTL cells
		5. Singe Cell Bar Coding (SCBC): 1x10^6 cells of pre-stimulated PBMC, QC sample and target cells for SCBC analysis.
		6. High Dimension Flow Cytometry – 2x10^6 cells of pre-stimulated PBMC, QC sample and target cells

# Shipping Instructions

* 1. All samples should be securely packaged in a container designed for shipping human biospecimens.
	2. Please refer the table at 4.0 for shipping condition for the **non-stimulated PBMC, QC samples and the target cells.**
	3. All samples may be shipped Monday-Thursday (non-holiday) by Federal Express for next day delivery (Tuesday-Friday)
	4. All sample labels should include the following information:
* On study ID number
* Center identification
* Collection date and study time-point
* Initials of the individual who collected the specimen

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Analysis** | **Recipient** | **Pre- stimulated****PBMC** | **QC Sample** | **Target Cells** | **Priority** | **Shipping instruction** |
| i. Single cell RNAseq analysis | Nationwide Children’sHospital | 2,000,000cells | 100,000 cells | 2,000,000cells | 1 | Cryopreserve the cells in 15% DMSO in 40% FBSin RPMI medium and ship batched samples in dry ice |
| ii. Nanostring Immunoprofiling | NationwideChildren’s Hospital | 500,000-1,000,000cells | 500,000-1,000,000cells | 500,000-1,000,000cells | 2 | Freeze the cell pellets in-80oC and ship batched samples in dry ice |
| iii. Mass Cytometry by Time of Flight(CyTOF) | Ohio State University | 1,000,000cells | 1,000,000cells | 1,000,000cells | 3 | ship fresh cells with ice pack |
| iv. T-cell Repertoire | New York Medical College | 2,000,000cells | 2,000,000cells | 300,000-500,000 cells | 4 | Freeze the cell pellets in-80oC and ship batched samples in dry ice |
| v. Single Cell Bar Coding (SCBC) | 1,000,000cells | 1,000,000cells | 1,000,000cells | 5 | Cryopreserve the cells in 10% DMSO in FBS and ship batched samples indry ice |
| vi. HighDimension Flow Cytometry | Children’sHospital of Pennsylvania | 2,000,000cells | 2,000,000cells | 2,000,000cells | 6 | Cryopreserve the cells in 10% DMSO in FBS andship batched samples in dry ice |

# Summary

1. **Detailed Shipping Address**
2. Nationwide Children’s Hospital

The Steve and Cindy Rasmussen Institute for Genomic Medicine Attn: Joyleen Oliver

Abigail Wexner Research Institute at Nationwide Children’s Hospital 575 Children’s Crossroad, WB2265

Columbus, OH 43215

Phone: 614-355-3589

Email: elaine.mardis@nationwidechildrens.org Contact before shipping

1. Nationwide Children’s Hospital

The Steve and Cindy Rasmussen Institute for Genomic Medicine Attn: Kristen Leraas

Abigail Wexner Research Institute at Nationwide Children’s Hospital

575 Children’s Crossroad, WB2265 Columbus, OH 43215

Phone: 614-355-3589

Email: elaine.mardis@nationwidechildrens.org Contact before shipping

1. Ohio State University

Nationwide Children’s Hospital Research Institute c/o Robin Nakkula, Dean Lee Lab

700 Childrens Dr WA 4112

Columbus, Ohio 43205

Phone: 614-355-1538

Email: Robin.Nakkula@nationwidechildrens.org Contact before shipping

1. New York Medical College Yaya Chu, PhD

Basic Science Building, Rm401 New York Medical College

40 Sunshine Cottage Road Valhalla, NY, 10595

Phone: 914-594-3726

email: yaya\_chu@nymc.edu Contact before shipping

1. New York Medical College Yaya Chu, PhD

Basic Science Building, Rm401 New York Medical College

40 Sunshine Cottage Road Valhalla, NY, 10595

Phone: 914-594-3726

email: yaya\_chu@nymc.edu Contact before shipping

1. Children’s Hospital of Pennsylvania Vella Lab

3501 Civic Center Blvd, Lab 10100

Philadelphia, PA 19104

Phone: 412-848-7461

Email: vellal@email.chop.edu

Contact before shipping

# APPENDIX 4: Characterization and Functional Assessment of the ex vivo expanded BKV CTL Product

1. **Objective**:

To characterize ex vivo expanded BKV CTL product (IFG+ cells from CCS product) (on all clinical products)

# Methods for Preparation of Cells

c. From the CCS viral CTL target cell fraction, remove 0.5-1e5 cells (50-100k cells), wash once by centrifugation, resuspend in 1 ml TexMACS supplemented with 100 IU/ml IL-2, and place in a 1 ml cryovial. Tightly seal the cryovial in preparation for **immediate overnight shipping** to the Johnson Lab at MCW (at 4 °C).

d. From the CCS CTL isolation non-target cell fraction, concentrate and wash the cells once by centrifugation, and resuspend in 15 ml TexMACS. Place the cell suspension in a 15 ml screw-cap conical centrifuge, and tighten the cap in preparation for **immediate overnight shipping** to the Johnson Lab at MCW (at 4 °C). These cells will be used to manufacture BLCL lines and as feeders to expand an aliquot of the target cell fraction in the Johnson Lab.

# Shipping Instructions

1. All samples should be securely packaged in a container designed for shipping human biospecimens.
2. The **freshly isolated non-target cells and target cells** (15 ml screw top conical and 1 ml cryovial, respectively, in a zip-lock bag) should be shipped overnight at 4 °C.
3. All sample labels should include the following information:
	* Patient study ID number
	* Center identification
	* Collection date and study time-point
	* Initials of the individual who collected the specimen

# Shipping Address

BMT Research Laboratory Attention: Huiqing Xu, MD

Froedtert Hospital Pavilion, Room 304 9200 West Wisconsin Avenue Milwaukee, WI 53226

Laboratory telephone: 414-805-6143

Call the BMT Research Laboratory at 414-805-6143 between the hours of 7:00 AM and 6:00 PM Central time to let them know a specimen is coming. Alternatively email:

palen@mcw.edu & james.weber@froedtert.com

Ship overnight express on the day of collection; Federal Express Account Number: TBD

Specimens will only be received Tuesday through Friday (except Holidays).

# APPENDIX 5: Detection of BKV CTL in the Blood of Patients Prior to Infusion of the BKV CTL Product

1. **Objectives**
* Determine whether detectable virus-specific T cells are present in the blood of CTL recipients prior to infusion of the CTL product
1. **Methods for Preparation of Cells**
	1. 15 ml of whole blood should be collected in a green top tube (sodium heparin) from the CTL **recipient** just prior to infusion of the CTL product.
	2. Please label the tube as “Pre-Infusion Sample”, along with the other information listed below.
	3. The blood sample should be shipped overnight at room temperature to the Johnson Lab at MCW.
2. **Shipping Instructions**
3. All samples should be securely packaged in a container designed for shipping human biospecimens.
4. The **whole blood sample** (green top tube) should be shipped overnight at room temperature.
5. All sample labels should include the following information:
	* Patient study ID number
	* Center identification
	* Collection date and study time-point
	* Initials of the individual who collected the specimen
6. **Shipping Address**

BMT Research Laboratory Attention: Huiqing Xu, MD

Froedtert Hospital Pavilion, Room 304 9200 West Wisconsin Avenue Milwaukee, WI 53226

Laboratory telephone: 414-805-6143

Call the BMT Research Laboratory at 414-805-6143 between the hours of 7:00 AM and 6:00 PM Central time to let them know a specimen is coming. Alternatively email: hxu@mcw.edu and/or fzhu@mcw.edu.

Ship overnight express on the day of collection; Federal Express Account Number: TBD Specimens will only be received Tuesday through Friday (except Holidays).

# APPENDIX 6: Biology Studies From Recipients Post-Infusion of Viral CTL

1. **Objective**

To investigate the immunological response in patients following viral CTLs derived from the Miltenyi CliniMACS Prodigy Gamma-capture system.

# Methods for Preparation of Cells

* 1. 20 ml of whole blood will be collected in a green top tube (sodium heparin) at each of the indicated time points below on patient (section 3.0).
	2. The blood samples should be shipped overnight at room temperature to the PCRF Laboratory at NYMC and PBMC will isolated and cryo- preserved at NYMC and distributed to other investigators for the following assays:
	3. high dimentional flow cytometry
	4. Lymphocyte proliferation as measured by CSFE
	5. Mass Cytometry by Time of Flight (CyTOF)
	6. Single cell bar code cytokine analysis
	7. TCR diversity and frequency by Immunoseq™
	8. Single Cell RNAseq
	9. Donor chimerism study

# Timing of Sample Collection

* Day 14 patient post-first infusion ± 3 days
* Day 60 patient post-last infusion ± 10 days
* Day 100 patient post-last infusion ± 10 days

# Shipping Instructions

1. All samples should be securely packaged in a container designed for shipping human biospecimens.
2. All **whole blood samples** (green top tubes) should be shipped overnight at room temperature.
3. All sample labels should include the following information:
	* Patient study ID number
	* Center identification
	* Collection date and study time-point
	* Initials of the individual who collected the specimen

# Shipping Address

Yaya Chu, PhD

Basic Science Building, Rm401 New York Medical College

40 Sunshine Cottage Road Valhalla, NY, 10595

email: yaya\_chu@nymc.edu. Lab 914-594-3726

Ship overnight express on the day of collection;

Specimens will only be received Tuesday through Friday (except Holidays).

# Appendix 7: Acute GVHD Grading (CIMBTR)



# APPENDIX 8. Evaluation and assessments of study procedures: flow-chart

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Screening | T-cell transfer | FU I | FU II |  |  |
| Visit I | II | III | IV | V | VI | VII | VIII | XI | X |
| Day –21 | Day 0 | Day 1 | Day 7 | Week 2 (±1d) | Week 4 (±2d) | Week 8 (±5d) | Week 12(±10d) | Day 180(±10d) | Day 365(±10d) |
| Patient informed consent | X |  |  |  |  |  |  |  |  |  |
| Inclusion / Exclusion Criteria | X |  |  |  |  |  |  |  |  |  |
| Demographiccharacteristics | X |  |  |  |  |  |  |  |  |  |
| Medical History | X |  |  |  |  |  |  |  |  |  |
| GVHD | X | Xa |  | Xa | Xa | Xa | **X**a | Xa | X | X |
| Hematologya | X |  | X | X | X | X | X | X |  |  |
| Chemistry | X |  |  |  |  |  |  |  |  |  |
| BK qRT-PCR | X, | X |  | Xa | Xa | Xa | Xa | Xa |  |  |
| T-cell Chimerism in third-party donors | X |  |  |  | X |  |  |  |  |  |
| Immune Studies | X | X |  |  |  |  | X | X | X | X |
| Physical examination | X |  |  |  |  |  |  |  |  |  |
| Vital signs | X |  |  |  |  |  |  |  |  |  |
| Performance Status | X |  |  |  |  | X | X | X | X | X |
| AEs/SAEs |  | X | X | X | X | X | X | X | X | X |
| Concomitant anti-viral medication | X | X | X | X | X | X | X | X |  |  |
| CTL Biology (Appendix III, IV, V, VI) | X | X |  |  | X |  | X | X |  |  |

a: weekly for 12 weeks post CTL infusion

***Appendix*** *9****:* NCI CTCAE v5.0 infusion-related reactions**

Infusion-related reaction is characterized by adverse reaction to the infusion of pharmacological or biological substances.

NCI CTCAE: National Cancer Institute Common Terminology Criteria for Adverse Events; NSAIDs: nonsteroidal anti- inflammatory drugs.

*Reproduced from: Common Terminology Criteria for Adverse Events (CTCAE), Version 5.0, November 2017, National Institutes of Health, National Cancer Institute. Available at: https://ctep.cancer.gov/protocoldevelopment/electronic\_applications/docs/CTCAE\_v5\_Quick\_Reference\_8.5x11.pdf*

# Appendix 10: Cytokine Release Syndrome Grading System\*

|  |  |
| --- | --- |
| Grade | Toxicity |
| Grade1 | Symptoms are not life threatening and require symptomatic treatment only, eg. Fever, nausea, fatigue, headache, mayalgias, malaise |
| Grade 2 | Symptoms require and respond to moderate interventionOxygen requirement <40% or hypotension responsive to fluids or low dose of one vasopressor |
| Grade 3 | Symptoms require and resond to aggressive interventionOxygen requirement >40% or hypotension requiring high dose or multiple vasopressors |
| Grade 4 | Life threatening symptomsRequirement for ventilator support |
| Grade 5 | Death |

* Lee DW, Gardner R, Porter DL, et al. Current concepts in the diagnosis and management of cytokine release syndrome. Blood 2014; 124: 188-195.

Appendix 11: Severe Chronic GVHD



Jagasia et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group Report. Biol Blood Marrow Transplant. 2015 March ; 21(3): 389–401.

**APPENDIX 12: Pre-existing HLA Antibodies study**

1. **Objective**

To investigate if pre-existing HLA anitbodies are in the recipients’ blood prior to the first viral CTL infusion.

1. **Methods for Preparation of Cells**
2. Peripheral Blood: Draw 3-5 mL of blood from the recipient prior to the first viral CTL infusion into a red top tube
3. Label the vacutainer tube with the patient’s study ID (patient number and patients’ initials), date and time of blood draw (dd-MM-yyyy format for the date (i.e., 01-JAN-03) and 24:00 hour clock format for the time).
4. Allow the blood to clot upright at room temperature for 30 minutes.
5. Rim the tubes with a wooden applicator stick and centrifuge the sample to isolate the serum supernatant) at 2800 x g for at least 10 minutes.
6. Draw off the supernatant and pipette 1.0 – 1.5 mL (1.5 mL MAX) of the serum into the properly labeled polypropylene specimen tube.
7. Freeze the samples in -80°C freezer until ready for shipment.
8. **Timing of Sample Collection**
	* Prior to the first viral CTL infusion to patient.
9. **Shipping Instructions**
10. All samples should be securely packaged in a container designed for shipping human biospecimens.
11. The isolated serum should be shipped overnight with dry ice.
12. All sample labels should include the following information:
	* Patient study ID number
	* Center identification
	* Collection date and study time-point
	* Initials of the individual who collected the specimen
13. **Shipping Address**

Yaya Chu, PhD

Basic Science Building, Rm401 New York Medical College

15 Dana road

Valhalla, NY, 10595

email: yaya\_chu@nymc.edu. Lab 914-594-3726

Please contact Dr. Cairo’s laboratory at (914) 594-3726 or email yaya\_chu@nymc.edu between the hours of 9:00 am and 5:00 pm EST Monday to Friday to inform the lab that a specimen is coming.