**A Pilot Study in the Treatment of Refractory Cytomegalovirus (CMV) Infections with Related Donor CMV Specific Cytotoxic T-cells (CTLs) in Children, Adolescents and Adult Recipients – NYMC 580**

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**A Pilot Study in the Treatment of Refractory Cytomegalovirus (CMV) Infections with Related Donor CMV Specific Cytotoxic T-cells (CTLs) in Children, Adolescents and Young Adult Recipients – NYMC 580**

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List of Abbreviations

AdV Adenovirus

Allo Allogeneic

BMT Bone marrow transplant

CMV 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

## 1.0 Study Hypothesis

CMV 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 cytomegalovirus (CMV) infection post Allogeneic Hematopoietic Stem Cell Transplantation (AlloHSCT), after solid organ transplant (SOT) or with primary immunodeficiencies (PID).

**2.0 Objectives**

2.1 Primary Objectives

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

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

2.2 Secondary Objectives

2.2.1 To determine the probability and severity of acute GVHD and chronic GVHD following CMV CTL administration in children, adolescents and adults with refractory CMV infection after AlloHSCT, after SOT or with PID.

2.2.2 To estimate the persistence of third-party CMV CTLs following CMV CTL administration in children, adolescents and adults with refractory viral infection(s) after AlloHSCT, after SOT or PID.

2.2.3 To determine the probability of 6 month and 1 year overall survival (OS) and viral-free survival (VFS) following CMV CTL administration in children, adolescents and adults with refractory CMV infection after AlloHSCT, after SOT or with PID.

2.2.4 To further investigate the genetic, proteomic and immunological properties of CMV CTLs derived from the Miltenyi CliniMACS Prodigy Gamma-capture system.

2.2.5 To quantitate specific cellular immune reconstitution and its correlation to antiviral response following CMV CTL infusions.

**3.0 Background and Rationale**

**3.1 Viral Infections Post-AlloHSCT**

Hematopoietic stem cell transplantation (HSCT) is curative therapy for many malignancies and non-malignant conditions. However, HSCT is associated with three major risks: graft rejection, graft-versus-host disease and opportunistic infections. Viral reactivation and infections remain a significant cause of morbidity and mortality in post-HSCT patients. These infections occur with delayed immune reconstitution, which may result from methods to reduce graft vs host disease (GVHD) such as *in vivo* serotherapy or *ex vivo* T depletion, and from GVHD itself. Although incidence and severity of viral infections/reactivations can be lowered by prophylactic and therapeutic antiviral antibiotics, the efficacy of this treatment is limited 1 2 3 4 5. Standard antiviral treatment does not lead to restored virus-specific immunity and thus, after therapy completion (usually day 100) new reactivations or infections are frequent. In addition, standard anti-viral antibiotics, including ganciclovir, foscarnet and cidofovir, are associated with significant side effects including leukopenia and renal dysfunction. Historic results of therapy for infections caused by Epstein-Barr virus (EBV), adenovirus (AdV) and cytomegalovirus (CMV) post HSCT have been dismal.

Previous investigations have shown that sufficient T-cell immunity is essential for the control and prevention of viral reactivations and newly occurring infections 6 7. For AdV-, CMV- and EBV-infections in particular, the development of virus-specific T-cell responses is associated with protection against virus-related complications post HSCT 8 9 10.

Monitoring of CMV by PCR and initiating appropriate pre-emptive therapy has reduced the risk of CMV disease 5. However, with the increased use of haploidentical donors, there is a high rate of CMV drug resistance with pre-emptive therapy. 11 Early mortality may be dependent upon the development of detectable CMV-specific CD4+ cells 9. The outcome of CMV reactivation/infection after HSCT is dependent on immune reconstitution as demonstrated in a study by Barron and colleagues; patients with high positive results in a lymphoproliferative assay (LPA) and higher counts for NK cell mediated responses had a lower incidence of viremia. Late onset CMV disease still results in high mortality rates 12, particularly in patients with delayed immune reconstitution 6.

Viral infections, including CMV, are also a cause of significant morbidity and mortality in patients with primary or secondary immunodeficiencies. Newborn screening (NBS) for severe combined immunodeficiency (SCID) may improve HSCT outcomes 13, but infants may become infected with CMV from nursing during the first few days before results of NBS are known. These infections may prove difficult to treat, with the absence of T cells, and many months before T cell engraftment post HSCT.

In summary, there is an urgent need for effective treatment of patients after HSCT or who have immunodeficiencies who suffer from systemic viral infections resistant to antiviral antibiotics and have insufficient immune reconstitution.

**3.2 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 14. 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. Different approaches of selection of virus-specific T cells have been studied for already nearly two decades 15. A recent review summarized the newer methodologies and results of virus-specific T cell therapy 1. Each offers advantages and each is associated with different challenges 16,17.

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 CMV, EBV and AdV uses repetitive re-stimulation of peripheral blood mononuclear cells with EBV-LCLs (lymphocyte cell lines) transduced with an Ad5f35pp65 vector 18. 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 19 20. 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.

**3.3 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.21 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.22-25 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.26-29

CMV infection is one of the most common infections after SOT, and may result in significant morbidity, mortality, and graft loss.30,31 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.32

Adenovirus (ADV) infections are more common in pediatric SOT recipients. 33,34 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.26-28 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.

**3.4 CliniMACS Cytokine Capture System, IFN-gamma**

**3.4.1 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 35. 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 Cytokine Capture System (IFN-) 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 36. Kinetics of the IFN-gamma response, cytotoxicity, alloreactivity and in-vitro expansion of the enriched cells have been investigated and analyzed thoroughly 37 38. The successful generation of multi-virus specific T cells after simultaneous incubation with several MACS GMP PepTivator peptide pools has also been demonstrated 39. Isolation of both CD8+ and CD4+ specific T cells help prevent immune escape of these viruses 40 41 42.

Clinical results and safety of the transfer of virus-specific T cells isolated and selected by as described above with the CliniMACS Plus system are available. Patients have been treated with CMV, adenovirus or EBV infections post HSCT 43 19 44 45.

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 35 46. The method was first described in 1999 15 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 47 . 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) 45.The successful enrichment of virus-specific T cells using the CliniMACS® Cytokine Capture System (IFN-) 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 36 .

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 36 37 39. The virus-specific T cells were used for patients with refractory CMV 48 49, AdV 43 or EBV infections 44 after HSCT.

**3.4.2 Clinical Studies using the CliniMACS Cytokine Capture System (IFN-)**

Peggs et al. report on the preemptive or prophylactic CMV-treatment of 18 patients after HSCT 45. Cell selection was successful in all cases using an older Miltenyi CliniMACS device, and the in vivo-expansion of CMV-specific T cells was observed within days after the adoptive transfer. Six of the seven patients treated prophylactically required no antiviral antibiotics throughout the study, although CMV infection occurred in one patient. Another patient who had been treated prophylactically remained free of infection for six months. Subsequently this patient required systemic steroid treatment for extensive chronic GVHD, and then developed CMV infection which required antiviral treatment. Of the 11 patients treated preemptively, nine received antiviral antibiotics against the initial viremia. Two patients treated preemptively did not need any antiviral antibiotics. Nine patients treated preemptively remained free of new CMV reactivations after clearing the first episode; two of these patients experienced new CMV reactivations subsequently. All were treated with 104 CMV-specific CD3+ cells/kg and followed up for 6 months. No infusional toxicities were observed. Acute GVHD grade 1° occurred in 5/18 patients; GVHD grade 2 was diagnosed in 2/18 patients, and acute GVHD grade 3 appeared in 1 patient, who received a T-cell replete HSC graft. Limited chronic GVHD was diagnosed in 3/18 patients whereas another 3/18 patients experienced extensive chronic GVHD (two of these patients received a T-cell replete transplant).

Feuchtinger et al.19 reported on the treatment of 18 patients (including 9 children) suffering from antibiotic refractory CMV disease or reactivation after HSCT from unrelated donors. Again, cell selection was successful in all cases. However, since the selection was for an extremely rare event, a minimum of 10% purity for IFN-+ cells was defined for product release. T-cell expansion *in vivo* was evaluable in 16 patients following the T-cell transfer. In 12 of these patients a successful T-cell response could be demonstrated within 4 weeks after adoptive T-cell transfer. Four patients failed to reach adequate anti-CMV T-cell levels. In contrast to the study described before, which explored prophylactic/preemptive treatment, the patients treated by Feuchtinger and colleagues all suffered from CMV-infections unresponsive to antiviral antibiotics. In 15 of 18 cases clearance of CMV viremia, or at least a 1 log reduction of viral load was observed. Non-responsiveness to the treatment was associated with a lack of T-cell expansion in two patients. The third non-responsive patient died of bacterial sepsis. Four of 18 patients in the study died of possibly CMV-related causes: three of the unresponsive patients who did not achieve an adequate T-cell response and one of the patients with initial successful T-cell expansion. No infusional toxicities were observed, and one case of mild GVHD was reported.

Meijet al generated 15 CMV-specific T-cell lines using the CliniMACS Cytokine Capture System 50 . Eight infusions were given to patients with refractory CMV reactivation. There were no adverse events, no GVHD, and CMV load disappeared.

In order to ensure rapid initiation of anti-viral T cells, a center in Germany has initiated GMP compliant manufacturing using the CliniMACS Cytokine Capture System 41 . Clinical data on safety and efficacy was obtained partly, and in the case of AdV infections, completely, from pediatric patients.

In summary, production of CMV cytotoxic T cells using this device has tremendous advantages compared to other methods. This processing method takes 24 hours following a donor apheresis, in contrast to other methods which take at least 7 days 51 52. This product will also contain both CD4 and CD8 T cells, which lowers the risk of viral evasion.

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

**3.5 Dose Justification of CMV CTLs**

Maximum dosages were based on previously published data. In a study on prophylactic/pre-emptive treatment of CMV infection patients received a target dose of 1×104 CD3+ cells/kg (range: 2.8×102 to 6.88×103 CD4+ cells/kg plus 6.0×101 to 3.99×103 CD8+ cells/kg)31. Acute GVHD occurred in 8 of 18 patients (grade 3 in 1 patient), limited chronic GVHD occurred in 3 of 18 patients and extensive chronic GVHD also occurred in 3 of 18 patients.

Feuchtinger et al.19 treated patients suffering from antibiotic-refractory CMV infection with a mean of 2.13×104 CD3+ cells/kg (matched and mismatched donors; range: 0.12×104 to 1.66×105 CD3+ cells/kg). One patient developed acute GVHD. In this setting, treatment success in terms of viral clearance was not related to the T-cell dose, and very low doses were sufficient to enable T cell in vivo-expansion. Moosmann et al. 44 used transfer of virus-specific T cells for treatment of patients suffering from EBV PTLD. Patients received a mean of 5.8×104 CD3+ cells/kg (range: 0.4×104 to 9.7×104 cells/kg). Three patients with late-stage PTLD at the time of T-cell transfer died despite treatment. No AEs related to the infused product have been reported for these patients, and possibility of GVHD occurrence cannot be assessed. In three early-stage patients remission of PTLD was observed. No GVHD occurred in these three patients. The mean cell number for adoptive transfer of EBV-specific T cells reported by Icheva et al was 5.8x103 /kg, ranging from 0.15 to 53x103 CD3+ cells/kg 18 . One of the patients responded to the lowest dose of 0.15x103 CD3+ cells/kg. Patients suffering from systemic AdV infection were treated in another pilot study by Feuchtinger et al. 43 with a mean of 1.4×104 CD3+ cells/kg (ranging from 0.12×104 to 5×104 CD3+ cells/kg). Patients clearing the infection in this study had received remarkable low numbers of virus-specific T cells (range: 0.12 to 0.6×104 CD3+ cells/kg). In one patient aggravation of pre-existing chronic GVHD of the skin was observed.

Thus, overall, pilot studies in 61 patients suffering from viral infections have shown promising therapeutic results after transfer of comparatively low numbers of specifically selected T cells and a highly satisfactory safety profile for such doses. Therapeutic doses chosen for the CMV-specific T cells are expected to provide the necessary treatment/prophylactic efficacy without raising safety problems. Furthermore, doses are adjusted for matched versus mismatched donors to reduce any risk of inducing GVHD in the latter setting. In this study the maximum dose is set at 2.5 × 104 T cells/kg for the virus-specific T cells from HLA matched donors and 0.5 × 104 T cells/kg for virus-specific T cells from HLA mismatched donors. No minimum thresholds are set. Of note, the threshold for GVHD is around 5 x 104/kg CD3+ so these doses are under this threshold.

## 4.0 Experimental Design

4.1 HLA Matched Related Donors: CMV 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).

4.2 HLA Mismatched Related Donors: CMV 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).

## 5.0 Patient and Donor Eligibility

**5.1 Patient Eligibility**

5.1.1 Patients with refractory CMV infection post allogeneic HSCT, post solid organ transplantation or with primary immunodeficiencies with either

- Increasing CMV 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:

- ANC < 500/mm2 secondary to ganciclovir

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

And/or

-known resistance to ganciclovir and/or foscarnet

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

5.1.3 Performance Status > 30% (Lansky < 16 yrs and Karnofsky > 16 yrs)

5.1.4 Age: 0.1 to 30.99 years (Cohort 1)

Age: 31 to 79.99 years (Cohort 2)

5.1.5 Females of childbearing potential with a negative urine pregnancy test at study entry only.

**5.2 Donor Eligibility**

5.2.1 Donor must be age 12 or greater.

5.2.1.1 Donors used for CTL recipients post allogeneic hematopoietic stem cell transplantation (HSCT) aged 12-18 years must be the same related sibling donor used for the allogeneic HSCT.

5.2.1.2 Donors used for pediatric CTL recipients post solid organ transplantation and/or primary immunodeficiencies, the donor must be ≥ 18 years of age.

5.2.2 Related donor available with a T-cell response CMV 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 PepTivators.

a. Third Party Allogeneic Donor (must be ≥ 18 years of age): If original donor is not available or does not have a T-cell response to CMV MACS® PepTivators: **third party related allogeneic donor** (family donor > 3 HLA A, B, DR match to recipient) with IgG positive to CMV and/or a T-cell response to the CMV MACS® PepTivator .

AND

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

AND

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

**5.3 Patient exclusion criteria:**

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

5.3.1 Patient with acute GVHD > grade 2 or severe chronic GVHD at the time of CMV CTL infusion

5.3.2 Patient receiving steroids (>0.5 mg/kg prednisone equivalent) at the time of CMV CTL infusion

5.3.3 Patient treated with donor lymphocyte infusion (DLI) within 4 weeks prior to CMV CTL infusion

5.3.4 Thymoglobulin (ATG) or Alemtuzumab within 30 days

5.3.5 Patient with poor performance status determined by Karnofsky (patients >16 years) or Lansky (patients ≤16 years) score ≤30%

5.3.6 CMV retinitis, meningitis, encephalitis, and/or cerebritis

5.3.7 Concomitant enrollment in another experimental clinical trial investigating the treatment of refractory CMV infection.

5.3.8 Any medical condition which could compromise participation in the study according to the investigator’s assessment

5.3.9 Known AIDS/uncontrolled HIV infection

5.3.10 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.

5.3.11 Known hypersensitivity to iron dextran

5.3.12 Patients unwilling or unable to comply with the protocol or unable to give informed consent.

5.3.13 Known human anti-mouse antibodies

## 6.0 Treatment

## 6.1 Study Overview

This open-label, phase I/II clinical trial will assess the safety and efficacy of CMV-specific CTLs isolated from whole blood or leukapheresis products. The CMV-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 (CMV).

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.

# 6.2 CMV CTLs: Dose and Administration:

**\*ALL PATIENTS MUST RECEIVE the FIRST CTL INFUSION AS AN INPATIENT AND BE MONITORED INPATIENT FOR ADVERSE EVENTS FOR A MINIMUM OF 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 6 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.**

6.2.1. Suspension of CMV-specific T cells in 10 mL of 0.9% NaCl with human serum albumin (HSA) given by IV bolus injection

6.2.2. HLA-identical related donors: Dose 2.5 × 104 CD3/kg recipient weight.

6.2.3. HLA mismatched related donors (mismatch at 1-5 antigens/alleles) Dose 0.5 × 104 CD3/kg recipient weight.

6.2.4 Additional doses of CMV CTLs a minimum of every 2 weeks (maximum 5 doses total)

6.2.4.1 - If recipients fail to respond to the first dose of CMV CTLs (qRT-PCR over the institutional level of upper normal) and have no acute or chronic GVHD and no persistent toxicities related to the past CTL infusions

6.2.5 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)

6.2.6 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).

## 6.3 Concomitant Medications

6.3.1. Antiviral Treatment

All patients will be additionally treated with antiviral chemotherapy as per local institutional standards: ganciclovir, foscarnet or valganciclovir. Change of second-line therapy to any of the above mentioned medications according to the investigator’s assessment is allowed. Prophylactic treatment with acyclovir is allowed throughout the study.

6.3.2. Prohibited medication and procedures

During the study, treatment with other investigational anti-CMV agents and treatment with donor lymphocyte infusions (DLIs) are prohibited in patients treated with the CMV-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 CMV CTL infusion.
  2. **CTL infusion will be held** if any one of the following is present:

A) ATG and/or alemtuzumab within 30 days

B) DLI within last 30 days

C) >grade II AGVHD

D) Patient receiving steroids (>0.5 mg/kg prednisone equivalent) at the time of CTL infusion.

E) Any dose limiting toxicity event (see 18.1.3) possibly, probably or definitely related to any CMV CTL prior infusion

F) Any grade 3-5 infusion-reaction, as graded by the NCI CTCAE v5.0, possibly, probably or definitely related to any CMV CTL prior infusion

G) Recipient seroconversion to any FDA-listed relevant communicable diseases which upon investigation, is determined to be caused or potentially caused by the CMV CTL infused.

H) Recipient septicemia is determined to be caused or potentially caused by contaminated CMV CTL infusion

I) Performance status less than 30%

If none of these criteria exist CMV-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.

**6.6** Management of Toxicity probably or definitely related to CMV CTLs

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

**7.0 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.

* 1. **Pre CTL Infusion Observations (Appendix III)**
     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. Plasma or Serum CMV 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. Immune Studies: Quantitative immunoglobulins and quantitative CD3+ CD4+, CD8+, CD19+ and CD3-/CD56+ peripheral blood
     10. All other laboratory monitoring according to the treating physician/standard of care
  2. **Post CMV CTLs Infusions Observations (Appendix III)**
     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 , and as clinically indicated (Appendix IV, Appendix VII)
     3. Plasma or serum CMV qRT-PCR for CMV 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 CMV 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. Persistence of CTLS: Donor chimerism will be obtained on day 14 (±3 days) after the first dose of CMV CTLs.
     7. Donor CBC, history and physical 7-14 days post CTL collection.

## 8.0 Preparation of the CMV CTLs

**8.1 Manufacture**

8.1.1. Manufacturing of the CMV 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.

8.1.1.1There is no blood drawn to manufacture CTLs, but following the leukapheresis, there is 100-150cc of mononuclear cells extracted. Additionally, approximately 100-150 cc of ACDA (citrate) is administered during leukapheresis and as such there is no volume loss.

8.1.1.2 Donors will be monitored for 4 hours post leukopheresis and they must have stable vital signs, be afebiles and be asymptomatic prior to discharge.

8.1.1.3 Donors will NOT receive any growth factors.

The individual, donor-derived blood product (whole blood or leukapheresis product) will be incubated with PepTivator® Peptide Pools of HCMV pp65. 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 CMV-specific T cells exceeds the number defined for the first dose of CMV-CTLs, the remaining CMV-CTLs will be cryopreserved. They may be given at a later time up to the defined total maximum dose, if necessary.

**8.1.2. Assessment**

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

**8.1.3. Release criteria for final products**

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

b. Among the CD4 and CD8 T cells, IFN+ cells target a goal of 10%; Local institutions may authority to do non-conforming release after approval from either Dr. Johnson (bjohnson@mcw.edu ) or Dr. Wang (WangY2@email.chop.edu).

c. Negative gram stain.

d. 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

## 8.1.4 Packaging, Labeling and Storage

8.1.4.1. Labeling

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

8.1.4.2. Storage

The CMV-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.

**9.0. STATISTICAL CONSIDERATIONS AND DATA ANALYSIS PLAN**

**9.1 Accrual and Duration**

We plan to investigate 40 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 month 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 CMV CTL infusion. For cohort 1, with 40 patients and 2 observed Grade 3/4 acute GVHD probably or directly related to CMV CTL infusions, the estimated Grade 3/4 GVHD rate will be 5% with 95% exact confidence interval (CI) [1%, 17%], which provides goodprecision for the estimated GVHD rate. For the efficacy endpoint, we expect that approximately 25% of patients will obtain a complete response to CMV-CTLs with undetectable viral load by qRT-PCR by week 12. With 40patients, this implies to 10 responses and the 95% exact CI for the 25% response rate will be [13%, 41%], again providing good 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 CMV CTL infusion i.e., when all patients have either completed Week 12 after CMV 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 CMV 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.

## 9.3 Analyses of the Primary Endpoints

### *9.3.1 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 CMV-CTL infusion after last CMV 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 CMV-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.

### *9.3.2 Efficacy*

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

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

9.3.2.2 Definition of Response to CMV-CTLs

Complete Response: CMV-PCR undetectable per lower limits of each institutional assay.

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

## 9.3.3 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 CMV 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 CMV 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.

## 9.3.4 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 CMV-CTLs and if necessary at 45 days after the sixth patients is infused with CMV-CTLs (Section 9.3.4) with particular attention to Grade III-IV acute GVHD probably or directly related to CMV-CTL infusion. These three patients have already been safely analyzed and will be included in the final 40cohort. 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.

### 9.4 Statistical Stopping Guidelines (Cohort 1)

9.4.1. 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 CMV-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 0.4% if the true grade III-IV acute GVHD event rate is 10%, stop early with a probability of 10.9% if the true event rate is 20%, stop early with a probability of 54.4% if the true event rate is 30%, and stop early with a probability of 91.9% if the true event rate is 40%. These probabilities are calculated from a simulation study.

|  |  |  |
| --- | --- | --- |
| Number of patients | Stop if grade >2  acute GVHD >= |  |
| 5-7 | 4 |  |
| 8-10 | 5 |  |
| 11-14 | 6 |  |
| 15-17 | 7 |  |
| 18-21 | 8 |  |
| 22-25 | 9 |  |
| 26-29 | 10 |  |
| 30-32 | 11 |  |
| 33-36 | 12 |  |
| 37-40 | 13 |  |

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

9.4.2. Infusional Toxicity, Hematopoietic Graft Failure and/or Cytokine Release Syndrome Probably or Directly Related to CMV-CTL Infusion

Infusional toxicity > grade 3 (NCI CTCAE v 5.0) (Appendix V), hematopoietic graft failure and CRS >grade 3 (Appendix VI) 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 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 0.6% if the true grade ≥ 3 infusional toxicity event rate is 5%, stop early with a probability of 11.9% if the true event rate is 10%, stop early with a probability of 87.0% if the true event rate is 25%, and stop early with a probability of 99.2% if the true event rate is 35%. These probabilities are calculated from a simulation study.

|  |  |  |
| --- | --- | --- |
| Number of patients | Stop if grade ≥ 3  infusional toxicity, hematopoietic graft failure or CRS >= |  |
| 5-8 | 3 |  |
| 9-14 | 4 |  |
| 15-21 | 5 |  |
| 21-27 | 6 |  |
| 28-34 | 7 |  |
| 35-40 | 8 |  |

9.4.3: 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 >2  acute GVHD >= |  |
| 5-7 | 4 |  |
| 8-10 | 5 |  |

9.4.4: Infusional Toxicity, Hematopoietic Graft Failure and/or Cytokine Release Syndrome Probably or Directly Related to CMV-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 ≥ 3  infusional toxicity, hematopoietic graft failure or CRS >= |  |
| 5-8 | 3 |  |
| 9-10 | 4 |  |

### 9.5.Overall survival rate (OS)

Overall survival is defined as time from CMV CTL infusion to death or last follow-up and will be assessed first at Day 1 and then throughout the study in each cohort 1 and 2.

## 9.6. 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).

**Definitions**

9.6.1 “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 CTL infusion.

9.6.2 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 CMV CTL infusion causing any one of the following complications:

9.6.2.1 Death,9.6.2.2 A life threatening adverse drug (cell therapy) experience,

9.6.2.3 Inpatient hospitalization or prolongation of existing hospitalization,

9.6.2.4 A persistent disability/incapacity,

9.6.2.5 A congenital anomaly/birth defect, or

9.6.2.6 Serious medical conditions defined as:

9.6.2.6.1 Grade 3-5 infusion reaction according to the NCI CTCAE v5.0 possibly, probably, or definitely related to CMV-CTL infusions within the first 24 hours after infusion

9.6.2.6.2 Recipient seroconversion to any FDA-listed relevant communicable diseases within 6 months of CMV-CTL infusion, which upon investigation, is determined to be caused or potentially caused by the CMV-CTLs;

9.6.2.6.3 Recipient bacteremia secondary to contaminated CMV-CTLs

9.6.2.6.4 Recipient develops any of the FDA listed relevant communicable diseases within 6 months of CMV-CTL infusion which upon investigation is determined to be caused or potentially caused by the CMV CTLs.

9.6.2.6.5 Any grade 3-5 adverse event considered probably, or definitely related to CMV-CTLs

9.6.3 Dose limiting criteria defined as:

9.6.3.1 Grade 3-5 infusion reaction, hematpoietic graft failure or CRS according to the NCI CTCAE v5.0 probably, or definitely related to CMV-CTLs infusions

9.6.3.2 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 CMV CTLs infusion

9.6.3.3 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.

**9.7 Reporting**

### 9.7.1 Reporting of Serious Adverse Events to the Sponsor/PI, Dr. Mitchell Cairo, and two study -Chairs, Dr. 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 CMV-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](mailto:jtalano@mcw.edu)) and Clinical Research Nurse (Lauren Harrison, RN [617-285-7844/lauren\_harrison@nymc.edu](mailto: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.

9.7.1.1 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.

**9.7.1.2 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.

### 9.7.1.3 Investigator Reporting to the FDA

9.7.1.3.1 Serious adverse events (SAEs) that are unlisted/unexpected, and either probably or directly related to CMV 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.**

**9.7.1.4** If the integrity of the CMV 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.

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

10.1 Removal from study secondary to CMV CTL grade III or IV infusion toxicity

10.2 Progression of CMV infection requiring new systemic anti-CMV therapy

10.3 Patient/Parent refusal to continue

10.4 Physician investigator determines it is not the best interest of the patient to continue therapy.

10.5 One year from Day 0 of the last CMV-CTL infusion.

10.6 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 CMV MACS® GMP PepTivator antigen(s) causing the therapy-refractory infection. The donor serology will also be assessed for CMV.

**1.0 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)

**2.0 Purpose:** The donor is evaluated to protect the safety of the recipient.

**3.0 Procedure:**

**3.1** **Determination of the allogeneic donor eligibility**

3.1.1 Responsibility of the transplant physician and is communicated to the collection and processing enter staff.

3.1.2 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;

3.1.3 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.

**3.2 Donor Health History Review**

3.2.1 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 screening for CJD and sepsis as part of the Donor History Questionnaire and the serological testing for IDMs includes West Nile Virus (WNV), HIV-1/2, hepatitis B, hepatitis C, HTLV I/II, Chagas, syphilis.

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* + 1. 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.

* + 1. 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.

* + - 1. Other atypical response identified

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

**3.4 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-CMV, CMV- urine
* CEBV serology
* Serologic test for syphilis
* West Nile Virus.
* Trypanosoma cruzi (Chagas’ Disease)

3.4.1 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:**

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1. Guidance for Industry: Eligibility Determination for Donors of Human Cells,

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**B. DONOR EVALUATION AND SCREENING FOR SUITABILITY**

**1.0 Principle:** Standards mandate criteria for allogeneic donor selection, evaluation, and management by trained medical personnel for both safety of the donor and recipient.

**2.0 Purpose:** The related donor is evaluated to protect the safety of the donor and recipient.

**3.0 Procedure**

**3.1 Suitability –** Applies to allogeneic donors.

**3.1.1** 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.

**3.1.1.2** Laboratory evaluation including CBC, chemistry panel, Mg, urinalysis, ABO and Rh.

**3.1.1.3** 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

**3.1.1.4** 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.

**3.1.1.5** Pregnancy assessment all female donors with childbearing potential within seven (7) days prior to collection.

**3.1.2** 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.

**3.1.3** Any abnormal finding of the prospective donor is documented in the in the donor record with recommendations made for follow-up care.

**3.1.4** Issues of donor health that pertain to the safety of the collection procedure are communicated in writing to the Collection Facility staff.

**3.2 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.

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**Appendix 2: Viral CTL Manufacturing**

**A. vCTL MANUFACTURING USING THE CliniMACS PRODIGY**

1. **Principle**
   1. 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.
   2. 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.
   3. 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.
   4. 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.
2. **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.

1. **Specimen**
   1. T cells, Apheresis
   2. T cells, Whole Blood
2. **Supplies Source**

4.1 Bag access Alaris

4.2 Syringes BD

4.3 Needles BD

4.4 Sterile Fields Medchoice

4.5 Alcohol wipes ITW Textwipe

4.6 Human serum albumin, 25% Pharmacy

4.7 Human serum albumin, 5% Pharmacy

4.8 CliniMACS PBS/EDTA buffer (3L) Miltenyi

4.9 CliniMACS PBS/EDTA buffer (1L) Miltenyi

4.10 Lymphocyte separation medium MP Biomedicals, LLC

4.11 50 ml polypropylene centrifuge tubes Sarstedt

4.12 50 ml polystyrene centrifuge tubes Corning

4.13 TexMACS GMP medium (2L) Miltenyi

4.14 MACS GMP Peptivator CMV pp65 (60nmol) Miltenyi

4.15 CliniMACS cytokine capture system Miltenyi

Containing: 7.5 mL CliniMACS IFN Catchmatrix Reagent

7.5 mL CliniMACS IFN Enrichment Reagent

4.16 0.9% Sodium Chloride as elution buffer Baxter

4.17 Prodigy TS500 tubing set Miltenyi

4.18 Research Peptivator CMV pp65 (6nmol) Miltenyi

4.19 IFN-gamma staining Miltenyi

4.20 Rapid Cytokine Inspector kit Miltenyi

4.21 Interleukin 2 (25g) Miltenyi

4.22 Pennicillin-Streptomycin Gibco

4.23 sterile water (250ml) Baxter

4.24 Pipets, individually wrapped Fisher

4.25 Plasma transfer sets with two couplers Fenwal

4.26 Transfer bags (1000ml, 600ml, 3000ml) Fenwal

4.27 Trypan Blue 0.4% in PBS Invitrogen

4.28 Pipet tips Fischer Scientific

4.29 Gloves SPD

4.30 15ml centrifuge tubes Corning

4.31 Tissue culture plates (24, 48well) Corning

4.32 Tissue culture flasks (vent cap) Corning

4.33 Cryotube vials (2ml) Nunc

1. **Equipment**

5.1 CliniMACS Prodigy device

5.2 Biological Safety Cabinet

5.3 Refrigerated centrifuge

5.4 COBE 2991

5.5 Pipet-aid

5.6 Pipettors 100-1000µl, 20-200µl, 2-20µl

5.7 CO2 incubators

5.8 Sebra heat sealer

5.9 Terumo sterile tubing welder

5.10 Microscope

5.11 ACT II diff Hematology Analyzer

5.12 Hemostats

5.13 FACSCalibur Flow cytometer

5.14 Remote monitoring camera

5.15 laptop

5.16 Hemacytometer

1. **Forms/Requisitions/Labels/Log Book**

6.1 Physician's order

6.2 Processing record review form

6.3 Acceptance of a stem cell product form

6.4 Blood Bank acceptance of a stem cell product (if applicable)

6.3 Stem Cell Product release form (for products infused fresh)

6.4 Infusion form (for products infused fresh)

6.5 vCTL manufacturing flowsheets

6.6 Microbiology requisitions

6.7 Blood Bank requisitions

6.8 Hematology requisitions

6.9 Endotoxin requisition form

6.10 LABS requisition form

6.11 Patient's identification labels

6.12 Intermediate labels

6.13 Certificate of analysis

1. **Procedure**

**vCTL manfacturing**

7.1 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.

7.2 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.

7.3 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.

7.3.1 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.

7.4 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.

7.5 Aliquot 1x109 total nucleated cells for the manufacturing process on the Prodigy.

7.5.1 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.

7.5.2 An apheresis product even with a high Hct generally does not require RBC reduction because the small volume needed to achieve 1x109 cells.

7.5.3 Final 1x109 cells are diluted and/or resuspended in 75ml of processing buffer before loading onto Prodigy.

7.6 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.

7.7 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:

7.7.1 Connections near valves 9, 10 and 12, and pre-column tubing placements.

7.7.2 Tubing flow around the pump is opposite of those on the CliniMACS Plus.

7.7.3 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.

7.7.4 The waste bag is to be left on the bench due to expected large fill volume.

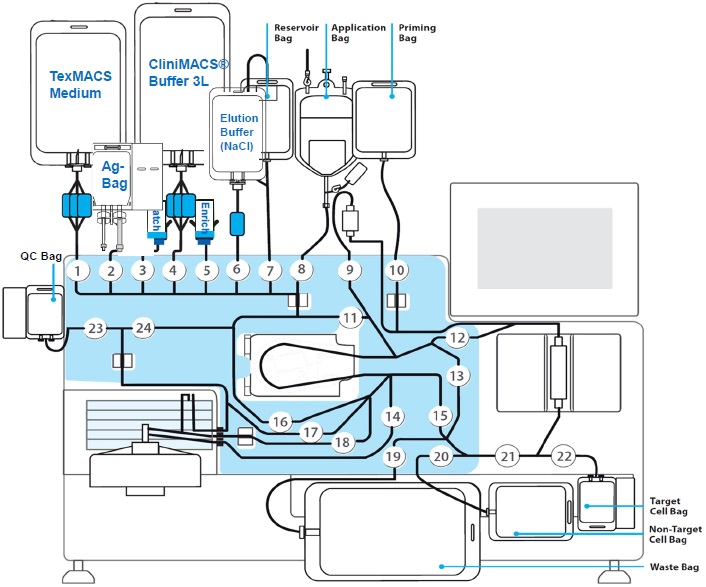
7.7.5 Tubing set must pass both the upper and lower part of integrity test.

7.8 After tubing set installation, the following steps are performed in order:

7.8.1 Connect TexMACS media, 3L processing buffer and elution buffer (0.9% NaCl).

7.8.2 Priming, which takes ~30min

7.8.3 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.



7.9 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.

7.10 Add reconstituted antigens(s)

7.10.1 Dissolve lyophilized Peptivator powder with 8ml of sterile water by directly injection into the vial. Mix to dissolve and minimize bubble formation.

7.10.2 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.

7.11 Connect CCS Catchmatrix and Enrichment reagents.

7.12 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.

7.12.1 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.

7.13 ~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.

7.13.1 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.

7.13.2 The QC bag contains a sample of 100ml Original fraction (ORI) of the pre-enriched cells. Perform cell count and flow analysis.

7.13.3 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+ cells. (See below)

7.14 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:

7.14.1 Add 0.5ml pre-chilled PBS/EDTA/0.5% HSA and centrifuge at 2700rpm x 5min.

7.14.2 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.

7.14.3 Add 1ml of RBC lysis solution to all 4 and incubate 10min at room temp.

7.14.4 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.

7.15 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.

7.15.1 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.

7.15.2 Use same gating strategy as described in Donor PreScreening for Virus Specific Cytotoxic T Lymphocytes.

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

7.15.3 The TC2 tube is for obtaining the viability of CD3+ cells.

7.16 The performance characteristic of the procedure is assessed by the following:

7.16.1 Recovery (CD4 or CD8) = # IFN cells (TC) **/** # IFN cells (ORI)

7.16.2 Corrected Recovery (CD4 or CD8) = # IFN cells (TC) .

# IFN cells (TC) **+** # IFN cells (NTC)

**** 7.16.3 Enrichment factor = % IFN cells (TC) % IFN cells (TC)

(CD4 or CD8) % IFN cells (ORI) % IFN cells (ORI)

7.17 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.

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.

7.18 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**

7.19 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.

7.20 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.

7.20.1 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.

7.20.2 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.

7.21 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.

7.21.1 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.

7.21.2 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.

7.22 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.

7.23 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.

1. **Expected Results**

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

.

**9.0 Quality Control Tests**

9.1 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.

9.2 Sterility testing is performed on the initial product and on the final product.

9.3 Endotoxin testing is performed on the final product.

9.4 CD4/IFN and CD8/IFN determination by Flow Cytometry is performed on the final product.

**10.0 References**

10.1 CliniMACS Prodigy CCS System User Manual. Miltenyi Biotec.

**B. 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.

1. **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**

Please collect 30 ml of peripheral blood in EDTA tubes on donor.

1. **Supplies and reagents Source**

4.1 PepTivator® CMV pp65 (6nmol) Miltenyi Biotec

4.2 TexMACS medium Miltenyi Biotec

4.3 Rapid Cytokine Inspector kit Miltenyi Biotec

4.4 IFN secretion assay detection kit Miltenyi Biotec

4.5 Phorbol 12-myristate 13-acetate (PMA) Sigma

4.6 Ionomycin calcium salt Sigma

4.7 Sterile Fields Medchoice

4.8 15 ml centrifuge tubes Corning

4.9 50 ml centrifuge tubes Corning

4.10 Tissue culture plate (24-well) Corning

4.11 12 x 75 mm test tubes BD Falcon

4.12 Gloves SPD

4.13 Pipets (5, 10, 25ml), individually wrapped Fisher Scientific

4.14 Pipettor tips 1-100 µl, 101-1000 µl Fisher

4.15 Sterile water Fisher

4.16 Microcentrifuge tubes Fisher

4.17 T25 tissue culture flask Nunc

4.18 Dimethyl sulfoxide (DMSO) Origen

4.19 Syringe (1cc) BD

4.20 Needles (18 gauge) BD

4.21 Alcohol wipes ITW Textwipe

4.22 Lymphocyte separation medium MP Biomedicals, LLC

1. **Equipment**

5.1 Biological Safety Cabinet

5.2 Refrigerated centrifuge

5.3 Pipet-aid

5.4 Pipettors 100-1000µl, 20-200µl, 2-20µl, 1-10µl

5.5 Microscope

5.6 ACT II diff Hematology Analyzer

5.7 Table top microcentrifuge

5.8 CO2 incubator

1. **Forms/Requisitions/Labels/Log Book**

6.1 Physician's order

6.2 Acceptance of a stem cell product form

6.3 Donor prescreening for vCTL flowsheet

6.4 Blood Bank requisitions

6.5 Virology requisitions

6.6 Patient's identification labels

6.7 Intermediate labels

1. **Procedure**
   1. Reconstitute the 6 nmol viral peptides (CMV) 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 CMV. 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.
   2. 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.
      2. 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 into 990) | 10μg/ml | 1:10 (10 into 5+85) | 1μg/ml |
| Ionomycin | 1mg/ml |  |  | 1:20 (5 into 85+10) | 50μg/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.
     2. 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.)
  3. 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).
  4. 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.
  5. **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.
  6. **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.
  7. **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.
  8. **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 |
| RCI cocktail | 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.

1. **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.

8.2 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

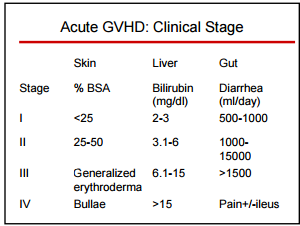
1. **Quality Control Tests**
   1. Nucleated cell counts and viability counts are performed on the initial product, at different points during the procedure as indicated in the SOP.
   2. CD4/IFN and CD8/IFN, determination by Flow Cytometry is performed on the post-stimulation product.
2. **References**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

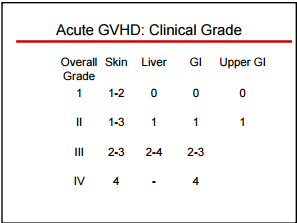
APPENDIXIII. Evaluation and assessments of study procedures: flow-chart

|  | 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 (±1 d) | Week 4 (±2 d) | Week 8 (±5 d) | Week 12  (±10 d) | Day 180  (±10 d) | Day 365  (±10 d) |
| Patient informed consent | X |  |  |  |  |  |  |  |  |  |
| Inclusion / Exclusion Criteria | X |  |  |  |  |  |  |  |  |  |
| Demographic characteristics | 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 |  |  |  |  |  |  |  |  |  |
| CMV 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 |  |  |

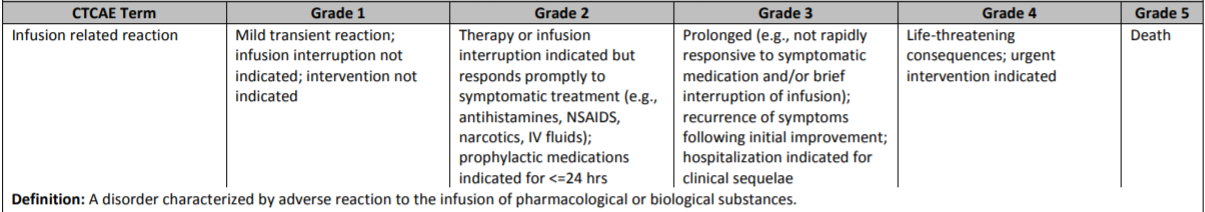
a: weekly for 12 weeks post CTL infusion

**Appendix IV: Acute GVHD Grading (CIMBTR)**





**Appendix V: 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 VI: 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 intervention  Oxygen requirement <40% or hypotension responsive to fluids or low dose of one vasopressor |
| Grade 3 | Symptoms require and resond to aggressive intervention  Oxygen requirement >40% or hypotension requiring high dose or multiple vasopressors |
| Grade 4 | Life threatening symptoms  Requirement 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 VII: 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.