**Single Patient Compassionate Use Treatment of Refractory Cytomegalovirus (CMV) Infections with Related Donor CMV Specific Cytotoxic T-cells (CTLs)**

**Institution name**: Riley Hospital for Children – Indiana University

**Sponsor:** April Rahrig, DO

Riley Hospital for Children

705 Riley Hospital Dr., Rm. 4340

Indianapolis, IN 46202

Phone: (317) 278-3014

Email: alrahrig@iu.edu

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 Background and Rationale**

**1.1 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.

**1.2 Immunologic therapy of viral infections**

Various methods for immunologic treatment of viral infections/reactivations after HSCT have been explored. Successful adoptive transfer of cytotoxic T-cell clones in bone marrow transplant recipients have been published as early as 1992 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.

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

**1.3.1 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.

**1.3.2 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.

**1.4 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.

**2.0 Brief Clinical History and Rationale for Use in this patient**

**Age:** 15 month old

**Gender:** Male

**Weight**: 10.7kg

**Allergies**: Topical chlorhexidine

**Diagnosis:** RAG1 mutation, SCID post umbilical cord stem cell transplant (with a prep of now with history of CMV disease now with recurrent CMV viremia

**Prior Therapy for CMV viremia:**

This patient had retinitis and pneumonitis, treated with IV Ganciclovir for 21 days, then transitioned to PO valganciclovir with continued fluctuation of CMV viremia. It was decided to proceed to stem cell transplant and IV ganciclovir was restarted to suppress viral load prior to stem cell transplant until Day 0. Prior to day 0, he had liver transaminitis which was thought to be a side effect of ganciclovir and was then transitioned to IV foscarnet during preparative regimen with improvement of transaminitis. He transitioned back to IV ganciclovir briefly for a week prior to Day 0 due to rise in CMV levels, however since his stem cell transplant (day 0) he has been on foscarnet IV, which is his current therapy. He is unable to be on IV ganciclovir due to his recent stem cell transplant and concern for myelosuppression and engraftment failure.

**Response to therapy (please reference table below):**

Initial CMV diagnosis in May 2023, he had serum CMB levels of up to 10,600 IU/ml. At this time he had retinitis and CMV pneumonitis. BAL revealed approximately 620,000 IU/ml of CMV. After 21 days of ganciclovir he was transitioned to PO valganciclovir and his serum CMV PCR levels ranged from 186 to 550IU/ml. When he was admitted for IV Ganciclovir prior to SCT his serum PCR level was 66 and ranged from 66 to 365 IU/ml prior to day 0. After he received his stem cell infusion his serum CMV PCR levels increased from 666 IU/ml on Day +2 post stem cell transplant, 11,600 IU/ml on day +6 and 5,070 IU/ml on Day +9. His CMV retinitis was diagnosed in May 2023, on his next ophthalmology exam in October 2023 and again on November 21, 2023 were negative for active CMV retinitis. His BAL was not repeated from his initial diagnosis in May 2023, but his respiratory symptoms improved from May and were at baseline prior to stem cell transplant. He currently has mild hypoxia and tachypnea which is likely related to fluid overload and mucositis post his stem cell transplant.

|  |  |  |
| --- | --- | --- |
| Date Range (Inpatient Admissions)  | Drug  | Viral Copies of CMV (range) IU/ml |
| 5/13/23-6/6/23 | IV Ganciclovir | 455-10,600 |
| 6/6-6/8 | PO Valganciclovir à DC home  | 243 |
| 6/21-6/27 | PO Valganciclovir à DC home | 186-550 |
| 10/2-10/19; admit for IV therapy due to lack of response and protective isolation pre-HSCT workup, switched due to LFT elevation to IV Foscarnet below | IV Ganciclovir | 66-204 |
| 10/19-11/7 | IV Foscarnet | 73-365 |
| 11/7-11/14  | IV Ganciclovir | 296-311 |
| 11/14- CURRENT  | IV Foscarnet  | 666 – 11,600 |

**Reason for request:**

I am requesting compassionate use of CMV viral specific T Cells due to this patient’s severe immunocompromised state, worsening of CMV viremia, the lack of current treatment options, and the risk of CMV dissemination, organ toxicity and even death. The two current FDA approved therapeutic options for patient’s this age are IV ganciclovir and IV foscarnet. He is not currently a candidate for IV ganciclovir at this time due to his recent stem cell transplant and the risk of engraftment failure and severe myelosuppression. Therefore, IV foscarnet is our only current treatment option, which he has been receiving. We are also giving him IVIG to potentially prevent dissemination of his current CMV viremia. This may help but is not expected to cure him. Resistance testing has been sent and is pending. If CMV resistance is detected the next therapeutic option would be Maribavir. Pediatric dosing of Maribavir has not been established and is not currently FDA indicated for a patients less than the age of 12 and less than 35kg. He does not qualify for this CMV viral specific TCell product on the clinical trial using this product given his history of CMV retinitis, even though it is not currently active based on his most recent ophthalmology exam. This CMV viral specific T Cell protocol is a derivation of a current clinical trial that has been approved, and my ask is for this product to be given as compassionate use. Therefore, in my professional opinion, CMV targeted viral specific T Cells are indicated to treat this patient’s CMV viremia and prevent worsening of his disease and potentially prevent mortality associated with his CMV viremia post stem cell transplant.

## 3.0 Patient and Donor Eligibility

**3.1 Patient Eligibility**

3.1.1 Patients with refractory CMV infection post allogeneic HSCT

- 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

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

3.1.3 Age: 0.1 to 30.99 years

3.1.4 >14 days from last ATG dose and >14 days from Stem cell infusion

**3.2 Donor Eligibility**

3.2.1 Related donor available with a T-cell response CMV MACS® PepTivators. As defined in Appendix 2, 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: 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 (Appendix 1).

AND

Obtained informed consents by donor.

## 4.0 Treatment

## 4.1 Study Overview

This protocol will use the 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).

# **4.2 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 may be discharged if afebrile and has normal heart rate, respiratory rate, blood pressure and is on room air. The patient will need to be seen daily for 5 more days as an outpatient 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 4.2.4 and 4.5) they may be administered outpatient per physician discretion monitoring per section 4.4 and observation up to 4 hours after the start of the infusion.**

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

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

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

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

4.2.4.1 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

4.2.5 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)

4.2.6 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).

## 4.3 Concomitant Medications

4.3.1 Antiviral Treatment

All patients will be additionally treated with antiviral chemotherapy as per local institutional standards: ganciclovir, foscarnet or valganciclovir.

4.3.2 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.

**4.4 Vital Signs**: temperature, blood pressure and heart rate will be obtained at 15, 30 60 and 120 minutes after each CMV CTL infusion.

**4.5 CTL infusion will be held** if any one of the following is present:

A) ATG and/or alemtuzumab within 14 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 7.0) 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.

**4.6** 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.

**5.0 Pre-Study Observations**

**5.1 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.

**5.2 Pre CTL Infusion Observations**

5.2.1 History and physical examination: A complete history and physical examination including weight, height, BSA.

5.2.2 Hematology (must be within one week prior to starting therapy): WBC, differential, platelet count.

5.2.3 Chemistry: Electrolytes, serum creatinine, BUN, total and direct bilirubin, SGPT(ALT), SGOT (AST), albumin, calcium, phosphorus, uric acid and magnesium, LDH.

5.2.4 Performance Status: Karnofsky or Lansky (age appropriate)

5.2.5 Baseline Chimerism Study: on CTL donor and recipient

5.2.6 Plasma or Serum CMV qRT-PCR

5.2.7 HLA typing: HLA A and B by intermediate resolution; DRB1 by high resolution on donor and recipient

5.2.8 Characterization on Validations product of CMV-CTLs (Appendix 3) (Minimum 3 validations per site) (only on manufacturing validations)

5.2.9 Characterization and Functional Assessment of the CMV CTL Clinical Grade Product(Appendix 4) (only on clinical product)

5.2.10 Pre-existing HLA Antibodies (Appendix 8)

5.2.11 All other laboratory monitoring according to the treating physician/standard of care

**5.3 Post CMV CTLs Infusions Observations**

5.3.1 CBC with manual differential, platelet count weekly (±3 days) through week 12 post last CTL infusion

5.3.2 GVHD: weekly assessment of stage and grade of both acute and chronic GVHD and as clinically indicated (Appendix 6 and 7)

5.3.3 Plasma or serum CMV qRT-PCR for CMV weekly (± 3 days) or more often if clinically needed, through week 12.

5.3.4 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

5.3.5 Performance Status: Karnofsky or Lansky to be documented at Day +30, 100, 180 and 365 (±10 days) post last CTL infusion.

## 6.0 Preparation of the CMV CTLs

**6.1 Manufacture**

6.1.1 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.

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.

**6.1.2 Assessment**

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

**6.1.3 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%

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

## 6.1.4 Packaging, Labeling and Storage

6.1.4.1 Labeling

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

6.1.4.2 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.

**7.0 Dose limiting criteria defined as:**

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

7.2 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

7.3 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.

**8.0 References**

 1. Bollard CM, Heslop HE: T cells for viral infections after allogeneic hematopoietic stem cell transplant. Blood 127:3331-40, 2016

 2. Lion T, Baumgartinger R, Watzinger F, et al: Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. Blood 102:1114-20, 2003

 3. Ocheni S, Kroeger N, Zabelina T, et al: EBV reactivation and post transplant lymphoproliferative disorders following allogeneic SCT. Bone Marrow Transplant 42:181-6, 2008

 4. Sandherr M, Einsele H, Hebart H, et al: Antiviral prophylaxis in patients with haematological malignancies and solid tumours: Guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society for Hematology and Oncology (DGHO). Ann Oncol 17:1051-9, 2006

 5. Zaia J, Baden L, Boeckh MJ, et al: Viral disease prevention after hematopoietic cell transplantation. Bone Marrow Transplant 44:471-82, 2009

 6. Lilleri D, Fornara C, Chiesa A, et al: Human cytomegalovirus-specific CD4+ and CD8+ T-cell reconstitution in adult allogeneic hematopoietic stem cell transplant recipients and immune control of viral infection. Haematologica 93:248-56, 2008

 7. Lilleri D, Gerna G, Fornara C, et al: Prospective simultaneous quantification of human cytomegalovirus-specific CD4+ and CD8+ T-cell reconstitution in young recipients of allogeneic hematopoietic stem cell transplants. Blood 108:1406-12, 2006

 8. Annels NE, Kalpoe JS, Bredius RG, et al: Management of Epstein-Barr virus (EBV) reactivation after allogeneic stem cell transplantation by simultaneous analysis of EBV DNA load and EBV-specific T cell reconstitution. Clin Infect Dis 42:1743-8, 2006

 9. Barron MA, Gao D, Springer KL, et al: Relationship of reconstituted adaptive and innate cytomegalovirus (CMV)-specific immune responses with CMV viremia in hematopoietic stem cell transplant recipients. Clin Infect Dis 49:1777-83, 2009

 10. Heemskerk B, Lankester AC, van Vreeswijk T, et al: Immune reconstitution and clearance of human adenovirus viremia in pediatric stem-cell recipients. J Infect Dis 191:520-30, 2005

 11. Shmueli E, Or R, Shapira MY, et al: High rate of cytomegalovirus drug resistance among patients receiving preemptive antiviral treatment after haploidentical stem cell transplantation. J Infect Dis 209:557-61, 2014

 12. Boeckh M, Leisenring W, Riddell SR, et al: Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. Blood 101:407-14, 2003

 13. Brown L, Xu-Bayford J, Allwood Z, et al: Neonatal diagnosis of severe combined immunodeficiency leads to significantly improved survival outcome: the case for newborn screening. Blood 117:3243-6, 2011

 14. Riddell SR, Watanabe KS, Goodrich JM, et al: Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science 257:238-41, 1992

 15. Brosterhus H, Brings S, Leyendeckers H, et al: Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. Eur J Immunol 29:4053-9, 1999

 16. Leen AM, Tripic T, Rooney CM: Challenges of T cell therapies for virus-associated diseases after hematopoietic stem cell transplantation. Expert Opin Biol Ther 10:337-51, 2010

 17. Leen AM, Heslop HE, Brenner MK: Antiviral T-cell therapy. Immunol Rev 258:12-29, 2014

 18. Icheva V, Kayser S, Wolff D, et al: Adoptive transfer of epstein-barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. J Clin Oncol 31:39-48, 2013

 19. Feuchtinger T, Opherk K, Bethge WA, et al: Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood 116:4360-7, 2010

 20. Koehne G, Hasan A, Doubrovina E, et al: Immunotherapy with Donor T Cells Sensitized with Overlapping Pentadecapeptides for Treatment of Persistent Cytomegalovirus Infection or Viremia. Biol Blood Marrow Transplant 21:1663-78, 2015

 21. Haidar G, Singh N: Viral infections in solid organ transplant recipients: novel updates and a review of the classics. Curr Opin Infect Dis 30:579-588, 2017

 22. Dharnidharka VR, Sullivan EK, Stablein DM, et al: Risk factors for posttransplant lymphoproliferative disorder (PTLD) in pediatric kidney transplantation: a report of the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS). Transplantation 71:1065-8, 2001

 23. Katz BZ, Pahl E, Crawford SE, et al: Case-control study of risk factors for the development of post-transplant lymphoproliferative disease in a pediatric heart transplant cohort. Pediatr Transplant 11:58-65, 2007

 24. Wistinghausen B, Gross TG, Bollard C: Post-transplant lymphoproliferative disease in pediatric solid organ transplant recipients. Pediatr Hematol Oncol 30:520-31, 2013

 25. Younes BS, McDiarmid SV, Martin MG, et al: The effect of immunosuppression on posttransplant lymphoproliferative disease in pediatric liver transplant patients. Transplantation 70:94-9, 2000

 26. Chiou FK, Beath SV, Wilkie GM, et al: Cytotoxic T-lymphocyte therapy for post-transplant lymphoproliferative disorder after solid organ transplantation in children. Pediatr Transplant 22, 2018

 27. Comoli P, Maccario R, Locatelli F, et al: Treatment of EBV-related post-renal transplant lymphoproliferative disease with a tailored regimen including EBV-specific T cells. Am J Transplant 5:1415-22, 2005

 28. Haque T, Wilkie GM, Jones MM, et al: Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. Blood 110:1123-31, 2007

 29. Khanna R, Bell S, Sherritt M, et al: Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. Proc Natl Acad Sci U S A 96:10391-6, 1999

 30. Gardiner BJ, Chow JK, Brilleman SL, et al: The impact of recurrent cytomegalovirus infection on long-term surivial in solid organ transplant recipients. Transpl Infect Dis Oct 3:e13189, 2019

 31. Kotton CN, Kumar D, Caliendo AM, et al: Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. Transplantation 96:333-60, 2013

 32. El Helou G, Rasonable RR: Safey considerations with current and emerging antiviral therapies for cytomegalovirus infection in transplantation. Expert Opin Drug Safe, 2019

 33. Boge CLK, Fisher BT, Petersen H, et al: Outcomes of human adenovirus infection and disease in a retrospective cohort of pediatric solid organ transplant recipients. Pediatr Transplant 23:e13510, 2019

 34. Florescu DF, Schaenman JM, Practice ASTIDCo: Adenovirus in solid organ transplant recipients: Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. Clin Transplant 33:e13527, 2019

 35. Bunos M, Hummer C, Wingenfeld E, et al: Automated isolation of primary antigen-specific T cells from donor lymphocyte concentrates: results of a feasibility exercise. Vox Sang 109:387-93, 2015

 36. Fahrendorff M vON, Rauser G et al: Automated generation of antigen-specific T cells for adoptive T cell therapy, 2010

 37. Gerdemann U, Keirnan JM, Katari UL, et al: Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. Mol Ther 20:1622-32, 2012

 38. Tischer S, Priesner C, Heuft HG, et al: Rapid generation of clinical-grade antiviral T cells: selection of suitable T-cell donors and GMP-compliant manufacturing of antiviral T cells. J Transl Med 12:336, 2014

 39. Richter A FA, Lasmanowicz V et al: Efficient and rapid in vitro generation of multi-virus-specific CD4+ and CD8+ T cells for adoptive immunotherapy., 2010

 40. Feuchtinger T, Lang P, Hamprecht K, et al: Isolation and expansion of human adenovirus-specific CD4+ and CD8+ T cells according to IFN-gamma secretion for adjuvant immunotherapy. Exp Hematol 32:282-9, 2004

 41. Zandvliet ML, Falkenburg JH, van Liempt E, et al: Combined CD8+ and CD4+ adenovirus hexon-specific T cells associated with viral clearance after stem cell transplantation as treatment for adenovirus infection. Haematologica 95:1943-51, 2010

 42. Zandvliet ML, van Liempt E, Jedema I, et al: Simultaneous isolation of CD8(+) and CD4(+) T cells specific for multiple viruses for broad antiviral immune reconstitution after allogeneic stem cell transplantation. J Immunother 34:307-19, 2011

 43. Feuchtinger T, Matthes-Martin S, Richard C, et al: Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol 134:64-76, 2006

 44. Moosmann A, Bigalke I, Tischer J, et al: Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. Blood 115:2960-70, 2010

 45. Peggs KS, Thomson K, Samuel E, et al: Directly selected cytomegalovirus-reactive donor T cells confer rapid and safe systemic reconstitution of virus-specific immunity following stem cell transplantation. Clin Infect Dis 52:49-57, 2011

 46. Rezaei N, Hedayat M, Aghamohammadi A, et al: Primary immunodeficiency diseases associated with increased susceptibility to viral infections and malignancies. J Allergy Clin Immunol 127:1329-41 e2; quiz 1342-3, 2011

 47. Campbell JD: Detection and enrichment of antigen-specific CD4+ and CD8+ T cells based on cytokine secretion. Methods 31:150-9, 2003

 48. Feuchtinger T, Lucke J, Hamprecht K, et al: Detection of adenovirus-specific T cells in children with adenovirus infection after allogeneic stem cell transplantation. Br J Haematol 128:503-9, 2005

 49. Kumaresan P, Figliola M, Moyes JS, et al: Automated Cell Enrichment of Cytomegalovirus-specific T cells for Clinical Applications using the Cytokine-capture System. J Vis Exp, 2015

 50. Meij P, Jedema I, Zandvliet ML, et al: Effective treatment of refractory CMV reactivation after allogeneic stem cell transplantation with in vitro-generated CMV pp65-specific CD8+ T-cell lines. J Immunother 35:621-8, 2012

 51. Naik S, Nicholas SK, Martinez CA, et al: Adoptive immunotherapy for primary immunodeficiency disorders with virus-specific T lymphocytes. J Allergy Clin Immunol 137:1498-1505 e1, 2016

 52. Papadopoulou A, Gerdemann U, Katari UL, et al: Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. Sci Transl Med 6:242ra83, 2014

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

* + 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:**

1. Foundation for the Accreditation of Cellular Therapy, Standards for Hematopoietic Progenitor Cell Collection, Processing and Transplantation, Sixth Edition 2015.

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

Tissues, and Cellular and Tissue-Based Products, U.S. Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research, August 2007

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

**1.0 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.

**REFERENCES:**

1. Foundation for the Accreditation of Cellular Therapy, Standards for Hematopoietic Progenitor Cell Collection, Processing and Transplantation, Sixth Edition 2015.

1. Confer, DL. Stroncek, DF. Bone Marrow and Peripheral Blood Stem Cell Donors.

In: Thomas Ed, Blume KG, Forman SJ (eds). Hematopoietic Cell Transplantation 2nd Ed. Malden, MA: Blackwell Science, Inc: 1999:421-430.

**Appendix 2: Viral CTL Manufacturing**

**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.

## APPENDIX 3: Validation Products

**1.0 Objective:**

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

1. **Methods for Preparation of Cells**
2. Each new center will perform 3 validations of either combination BKV, CMV, ADV or EBV CTLs.
3. The 5-6 million cells of pre-stimulated donor PBMC, QC samples from Prodigy and the final validation products will be needed for the following studies:

i. single cell RNAseq analysis- 1x10^5 cells of pre-stimulated PBMC, QC sample and target cells

ii. Nanostring Immunoprofiling: 5x10^5 to 1x10^6 cells from pre-stimulated PBMC, QC sample and target cells

iii. Mass Cytometry by Time of Flight (CyTOF): 1-2 x10^6 cells of pre-stimulated PBMC, QC sample and target cells

iv. T-cell Repertoire: 2x10^6 cells of pre-stimulated PBMC and QC sample and 3x10^5 target CTL cells

v. Singe Cell Bar Coding (SCBC): 1x10^6 cells of pre-stimulated PBMC, QC sample and target cells for SCBC analysis.

vi. High Dimension Flow Cytometry – 2x10^6 cells of pre-stimulated PBMC, QC sample and target cells

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

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

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

The Steve and Cindy Rasmussen Institute for Genomic Medicine

Attn: Joyleen Oliver

Abigail Wexner Research Institute at Nationwide Children’s Hospital

575 Children’s Crossroad, WB2265

Columbus, OH 43215

Phone: 614-355-3589

Email: elaine.mardis@nationwidechildrens.org

Contact before shipping

1. Nationwide Children’s Hospital

The Steve and Cindy Rasmussen Institute for Genomic Medicine

Attn: Kristen Leraas

Abigail Wexner Research Institute at Nationwide Children’s Hospital

575 Children’s Crossroad, WB2265

Columbus, OH 43215

Phone: 614-355-3589

Email: elaine.mardis@nationwidechildrens.org

Contact before shipping

1. Ohio State University

Nationwide Children’s Hospital Research Institute

c/o    Robin Nakkula, Dean Lee Lab

700 Childrens Dr WA 4112

Columbus, Ohio  43205

Phone: 614-355-1538

Email: Robin.Nakkula@nationwidechildrens.org

Contact before shipping

1. New York Medical College

Yaya Chu, PhD

    Basic Science Building, Rm401

    New York Medical College

    40 Sunshine Cottage Road

    Valhalla, NY, 10595

Phone: 914-594-3726

    email: yaya\_chu@nymc.edu

Contact before shipping

1. New York Medical College

Yaya Chu, PhD

    Basic Science Building, Rm401

    New York Medical College

    40 Sunshine Cottage Road

    Valhalla, NY, 10595

Phone: 914-594-3726

    email: yaya\_chu@nymc.edu

Contact before shipping

1. Children’s Hospital of Pennsylvania

Vella Lab

3501 Civic Center Blvd,

Lab 10100

Philadelphia, PA 19104

Phone: 412-848-7461

Email: vellal@email.chop.edu

Contact before shipping

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

**1.0 Objective**:

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

1. **Methods for Preparation of Cells**
2. From the CCS viral CTL target cell fraction, remove 0.5-1e5 cells (50-100k cells), wash once by centrifugation, resuspend in 1 ml TexMACS supplemented with 100 IU/ml IL-2, and place in a 1 ml cryovial. Tightly seal the cryovial in preparation for **immediate overnight** **shipping** to the Johnson Lab at MCW (at 4 °C).
3. From the CCS CTL isolation non-target cell fraction, concentrate and wash the cells once by centrifugation, and resuspend in 15 ml TexMACS. Place the cell suspension in a 15 ml screw-cap conical centrifuge, and tighten the cap in preparation for **immediate overnight** **shipping** to the Johnson Lab at MCW (at 4 °C). These cells will be used to manufacture BLCL lines and as feeders to expand an aliquot of the target cell fraction in the Johnson Lab.
4. **Shipping Instructions**
5. All samples should be securely packaged in a container designed for shipping human biospecimens.
6. The **freshly isolated non-target cells and target cells** (15 ml screw top conical and 1 ml cryovial, respectively, in a zip-lock bag) should be shipped overnight at 4 °C.
7. All sample labels should include the following information:
	* + Patient study ID number
		+ Center identification
		+ Collection date and study time-point
		+ Initials of the individual who collected the specimen
8. **Shipping Address**

BMT Research Laboratory

Attention: Huiqing Xu, MD

Froedtert Hospital Pavilion, Room 304

9200 West Wisconsin Avenue

Milwaukee, WI 53226

Laboratory telephone: 414-805-6143

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

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

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

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

**Appendix 6: Acute GVHD Grading (CIMBTR)**





**Appendix 7: Severe Chronic GVHD**



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

**APPENDIX 8: Pre-existing HLA Antibodies study**

**1.0 Objective**

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

1. **Methods for Preparation of Cells**

1. Peripheral Blood: Draw 3-5 mL of blood from the recipient prior to the first viral CTL infusion into a red top tube

2. Label the vacutainer tube with the patient’s study ID (patient number and patients’ initials), date and time of blood draw (dd-MM-yyyy format for the date (i.e., 01-JAN-03) and 24:00 hour clock format for the time).

3. Allow the blood to clot upright at room temperature for 30 minutes.

4. Rim the tubes with a wooden applicator stick and centrifuge the sample to isolate the serum supernatant) at 2800 x g for at least 10 minutes.

5. Draw off the supernatant and pipette 1.0 – 1.5 mL (1.5 mL MAX) of the serum into the properly labeled polypropylene specimen tube.

6. Freeze the samples in -80°C freezer until ready for shipment.

1. **Timing of Sample Collection**
* Prior to the first viral CTL infusion to patient.
1. **Shipping Instructions**
2. All samples should be securely packaged in a container designed for shipping human biospecimens.
3. The isolated serum should be shipped overnight with dry ice.
4. All sample labels should include the following information:
	* + Patient study ID number
		+ Center identification
		+ Collection date and study time-point
		+ Initials of the individual who collected the specimen
5. **Shipping Address**

 Yaya Chu, PhD

    Basic Science Building, Rm401

    New York Medical College

    15 Dana road

    Valhalla, NY, 10595

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

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

**1.0 Objective**:

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

1. **Methods for Preparation of Cells**
2. From the CCS viral CTL target cell fraction, remove 0.5-1e5 cells (50-100k cells), wash once by centrifugation, resuspend in 1 ml TexMACS supplemented with 100 IU/ml IL-2, and place in a 1 ml cryovial. Tightly seal the cryovial in preparation for **immediate overnight** **shipping** to the Johnson Lab at MCW (at 4 °C).
3. From the CCS CTL isolation non-target cell fraction, concentrate and wash the cells once by centrifugation, and resuspend in 15 ml TexMACS. Place the cell suspension in a 15 ml screw-cap conical centrifuge, and tighten the cap in preparation for **immediate overnight** **shipping** to the Johnson Lab at MCW (at 4 °C). These cells will be used to manufacture BLCL lines and as feeders to expand an aliquot of the target cell fraction in the Johnson Lab.
4. **Shipping Instructions**
5. All samples should be securely packaged in a container designed for shipping human biospecimens.
6. The **freshly isolated non-target cells and target cells** (15 ml screw top conical and 1 ml cryovial, respectively, in a zip-lock bag) should be shipped overnight at 4 °C.
7. All sample labels should include the following information:
	* + Patient study ID number
		+ Center identification
		+ Collection date and study time-point
		+ Initials of the individual who collected the specimen
8. **Shipping Address**

BMT Research Laboratory

Attention: Huiqing Xu, MD

Froedtert Hospital Pavilion, Room 304

9200 West Wisconsin Avenue

Milwaukee, WI 53226

Laboratory telephone: 414-805-6143

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

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

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

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