A Pilot Study in the Treatment of Refractory Epstein-Barr Virus (EBV) Infection with Related Donor EBV Cytotoxic T-Lymphocytes in Children, Adolescents and Adult Recipients – NYMC 581

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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
SOT	Solid organ transplant

1.0 Study Hypothesis

Related donor Epstein-Barr Virus (EBV) specific cytotoxic T cells (CTLs) manufactured with the Miltenyi CliniMACS Prodigy Cytokine Capture System will be safe and effective in decreasing EBV load in children, adolescents and adults with refractory EBV infection post Allogeneic Hematopoietic Stem Cell Transplantation (AlloHSCT), solid organ transplant (SOT) or with primary immunodeficiencies (PID).

2.0 Objectives

2.1 Primary Objectives

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

2.1.2 To estimate the efficacy of related donor EBV specific T cells (CTLs) in the treatment of children, adolescents and adults with refractory EBV infections after allogeneic HSCT, 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 EBV CTL administration in children, adolescents and adults with refractory EBV infection after AlloHSCT, SOT or with PID.

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

2.2.4 To further investigate the genetic, proteomic and immunological properties of related donor EBV-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 EBV 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].

EBV infection leading to post-transplantation lymphoproliferative disorder (PTLD) is a serious complication post both HSCT and solid organ transplant (SOT) [8] , [3] , [11]. The pediatric age group is at high risk, as many children are EBV negative, and many receive grafts from EBV positive donors. Other risk factors include the use of *in vivo* and *ex vivo* T depletion (e.g., use of ATG *or ex vivo* T depleted hematopoietic stem cell grafts). Before treatment with rituximab became available, most patients who developed PTLD died [12] , [11] . Pre-emptive therapy with rituximab is generally recommended for patients with EBV infections. However, in a recent review a response rate to rituximab treatment of only 63% in patients with PTLD was reported [13]. In a study published by Ocheni and colleagues 7 out of 9 patients died despite treatment with rituximab [3]. The use of rituximab often results in prolonged B cell aplasia, requiring long term IVIG replacement.

Viral infections are also a cause of significant morbidity and mortality in patients with primary or secondary immunodeficiencies. These infections may prove difficult to treat, with the absence of T cells, and many months before T cell engraftment post HSCT.

EBV can result in refractory lymphoproliferative disease in patients with Wiskott-Aldrich syndrome, X-linked lymphoproliferative syndrome (XLP), common variable immunodeficiency and hyper-IgM syndrome [14]. While these diseases can be cured with HSCT, EBV specific CTLs may also help treat and decrease morbidity and mortality while waiting for T cell engraftment [15] , [16] .

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 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.[17] 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.[18-21] 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.[22-25]

CMV infection is one of the most common infections after SOT, and may result in significant morbidity, mortality, and graft loss.[26,27] 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.[28]

Adenovirus (ADV) infections are more common in pediatric SOT recipients. [29,30] 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.[22-24] 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.

3.3 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 [31]. 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 [32]. 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 [33] [34].

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 [35] . 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 [36] [37]. 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.

3.4 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 [38]. 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 [39]. Kinetics of the IFN-gamma response, cytotoxicity, alloreactivity and in-vitro expansion of the enriched cells have been investigated and analyzed thoroughly [40], [41]. The successful generation of multi-virus specific T cells after simultaneous incubation with several MACS GMP PepTivator peptide pools has also been demonstrated [42]. Isolation of both CD8+ and CD4+ specific T cells help prevent immune escape of these viruses [43] [44] [45].

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 [46] [36], [47], [48].

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 [38] [42]. The method was first described in 1999 [32] 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 [49]. 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) [48]. 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 [39].

The CliniMACS® Prodigy which will be used in this study is a newly developed system for the fully automated selection and isolation of multivirus-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 [15], [40], [42]. The virus-specific T cells were used for patients with refractory CMV [46], [50], AdV [46] or EBV [47] infections 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 ([48]. 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 10^4 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. [36] 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.

Nine pediatric patients with systemic AdV infection after allogeneic HSCT were treated with adoptive T-cell transfer in another pilot study reported by Feuchtinger et al. [46] Three patients were not evaluable for the *in vivo*-expansion of the T cells transferred because they died too soon after adoptive T-cell transfer. One patient relapsed and was subsequently treated with high dose steroids and chemotherapy. One patient died of pre-existing multi-organ failure which had been caused by the AdV infection. One patient died one day after adoptive T-cell transfer of pre-existing myocardial infarction.

In 5 of the remaining 6 patients, stable in vivo-expansion of AdV-specific T cells after adoptive transfer could be demonstrated. One of the 6 patients evaluable for T-cell expansion did not achieve a successful T-cell response and died subsequently of generalized AdV infection. One of the patients who had a successful T-cell response subsequently died of multiorgan failure due to the AdV infection. However, 4 of 9 patients treated achieved AdV clearance. None of the treated patients showed infusional toxicities after administration of virus-specific T cells, and no case of acute GVHD was reported. In one patient, GVHD improved from grade 4 to grade 2 after the administration of the specific T cells. One patient experienced an aggravation of a pre-existing chronic GVHD of the skin.

Feucht et al. treated 30 pediatric patients with AdV specific T cells for patients with AdV refractory to cidofovir [51]. Refractory infection was defined as persistent number of AdV copies detected by PCR despite appropriate anti-viral antibiotics for 2 weeks. Changes in copy number were defined as $\geq$1 log change. Patients received AdV specific T cells and enrichment of IFN-□-secreting cells performed using the Miltenyi ClniMACS cytokine secretion system. A minimum of 10% purity for IFN-□+ cells was also used as a release criterion. In 61% (14) of 23 evaluable patients, there was successful in vivo expansion of AdV specific T cells. Cryropreserved T cells also led to a virologic response. Of note, patients treated with lymphocyte depleting agents (ATG, alemtuzumab) within 50 days of transplant had no in vivo expansion of T cells.

Responses: 21 complete clearance, 3 with >1 log decrease in copy numbers, and 4 with clearance of AdV infection at specific sites. Eight patients had no response. There did not appear to be a difference in response among those treated with or without cidofovir during anti-viral T cell therapy. All non-responders died as a result of AdV infections, in contrast to 71% of responders. However, causes of death in these patients were not related to AdV. Two patients developed grade 1 GVHD following anti-viral therapy, but these patients had preceding GVHD and had steroids weaned prior to anti-viral cellular infusion.

Rapid and stable expansion of T cells transferred could also be demonstrated by Moosmann et al. [47] who treated 6 post-HSCT patients suffering from EBV PTLD with adoptive EBV-specific T-cell transfers. In 3 of these patients who had early-stage disease at the time of transfer complete and stable remission of PTLD was achieved. No side effects associated with the infusion and no GVHD were observed in these three patients. Three patients who had late-stage PTLD with multi-organ dysfunction at the time of the transfer could not clear the disease and subsequently died of multi-organ

failure. Two of the patients with PTLD were alive and well at the end of the study, and have remained free of EBV-associated disease throughout the follow-up for more than 2 years. One died of idiopathic pneumonitis unrelated to EBV infection 2 months after the adoptive transfer.

Icheva et al [35] were able to demonstrate improvement with EBV-specific T cells. Ten patients (6 children) suffering from EBV viremia and/or PTLD were treated with EBV-specific T cells. Eight of ten treated patients responded with an in-vivo expansion of EBV-specific T cells. Seven of ten patients also had a clinical response to the adoptive T-cell transfer. No acute toxicities were observed following the T-cell transfer. Two patients developed new GVHD (Grade 1 skin GVHD). In one patient, Grade 2 gut GVHD resolved following the T-cell transfer. In another patient, pre-existing Grade 1 GVHD of the skin persisted. In two patients, pre-existing GVHD increased: one patient suffering from Grade 2 gut GVHD and Grade 3 liver GVHD developed Grade 4 GVHD, which was thought to be caused by the discontinuation of immunosuppression. The second patient suffered from Grade 1 GHVD prior to the T-cell transfer and developed Grade 3-4 gut GVHD eight weeks after the T-cell transfer. Two of the non-responders died due to the underlying PTLD. Out of the seven patients who had a clinical response to the treatment, three were in complete remission until the last observation, three died of other infectious complications and one patient died as a result of relapse of malignancy.

Meij et al generated 15 CMV-specific T-cell lines using the CliniMACS Cytokine Capture System [52]. 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 [44]. Clinical data on safety and efficacy was obtained partly, and in the case of AdV infections, completely, from pediatric patients.

In summary, production of viral 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 [16] , [53]. 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 anti-viral CTLs

In this first study with multi-virus-specific T cells, 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 1×10^4 $CD3^+$ cells/kg (range: 2.8×10^2 to 6.88×10^3 $CD4^+$ cells/kg plus 6.0×10^1 to 3.99×10^3 $CD8^+$ cells/kg) [3]. 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. [36] treated patients suffering from antibiotic-refractory CMV infection with a mean of 2.13×10^4 $CD3^+$ cells/kg (matched and mismatched donors; range: 0.12×10^4 to 1.66×10^5 $CD3^+$ 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. [47] used transfer of virus-specific T cells for treatment of patients suffering from EBV PTLD. Patients received a mean of 5.8×10^4 $CD3^+$ cells/kg (range: 0.4×10^4 to 9.7×10^4 cells/kg). Three patients with late-stage PTLD at the time of T-cell transfer died despite treatment. No adverse events 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 $5.8x10^3$ /kg, ranging from 0.15 to $53x10^3$ $CD3^+$ cells/kg [35]. One of the patients responded to the lowest dose of $0.15x10^3$ $CD3^+$ cells/kg. Patients suffering from systemic AdV infection were treated in another pilot study by Feuchtinger et al. [46] with a mean of 1.4×10^4 $CD3^+$ cells/kg (ranging from 0.12×10^4 to 5×10^4 $CD3^+$ cells/kg). Patients clearing the infection in this study had received remarkable low numbers of virus-specific T cells (range: 0.12 to 0.6×10^4 $CD3^+$ 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 virus-specific T cells are expected to provide the necessary treatment/prophylactic efficacy without raising safety problems. Furthermore, doses are adjusted for HLA matched versus mismatched donors to reduce any risk of inducing GVHD in the latter setting. In this study the maximum dose is set at 2.5×10^4 T cells/kg for the virus-specific T cells from HLA matched donors and 0.5×10^4 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 10^4/kg CD3+ so these doses are under this threshold.

4.0 Experimental Design

4.1 HLA Related Matched Donors: Epstein-Barr virus CTLs (2.5 x 10^4 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 10^4 CD3/kg).

4.2 HLA Mismatched Related Donors: Epstein-Barr virus CTLs (0.5x10^4 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 10^4 CD3/kg).

5.0 Patient and Donor Eligibility

5.1 Patient Eligibility

5.1.1 Patients with Epstein-Barr virus infections post allogeneic HSCT, post solid organ transplant or with primary immunodeficiencies with:

- Increasing EBV RT-PCR DNA (from baseline) after 7 days or persistent quantitative EBV RT-PCR DNA copies after 14 days despite two weeks of appropriate anti-viral therapy

and/or

-progressive clinical symptoms attributable to EBV, including biopsy proven colitis, lymphadenopathy, hepatomegaly, splenomegaly

AND/OR

- Medical intolerance to anti-viral therapies including:

-intolerance to rituximab

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 $\leq$ 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 Related donor available with a T-cell response EBV 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 EBV PepTivators.

a. Third Party Related Allogeneic Donor: If original donor is not available or does not have a T-cell response to EBV MACS® PepTivator: **third party related allogeneic donor** (family donor ≥ 3 HLA A, B, DR match to recipient) with IgG positive to EBV and/or a T-cell response at least to the EBV MACS® PepTivator.

AND

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

AND

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

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 CTL infusion
5.3.2. Patient receiving steroids (>0.5 mg/kg prednisone equivalent) at the time of CTL infusion
5.3.3. Patient treated with donor lymphocyte infusion (DLI) within 4 weeks prior to CTL infusion
5.3.4. Patient with poor performance status determined by Karnofsky (patients >16 years) or Lansky (patients ≤16 years) score ≤30%
5.3.5. Concomitant enrollment in another experimental clinical trial investigating the treatment of refractory EBV infection
5.3.6. Any medical condition which could compromise participation in the study according to the investigator's assessment
5.3.7. Known AIDS/uncontrolled HIV infection
5.3.8 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.
5.3.9 Known hypersensitivity to iron dextran
5.3.10 Patients unwilling or unable to comply with the protocol or unable to give informed consent.
5.3.11 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 related donor EBV-specific CTLs isolated from whole blood or leukapheresis products. The EBV-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 EBV Select (containing among other antigens, NA-1, LMP2A and BZLF-1) for enrichment.

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 Epstein-Barr virus CTLs: Dose and Administration:

ALL PATIENTS MUST RECEIVE the FIRST CTL INFUSION AS AN INPATIENT AND BE MONITORED INPATIENT FOR ADVERSE EVENTS FOR A MINIMUNE OF 24 HOURS DAYS FOLLOWING the CTL INFUSION

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

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

6.2.1. Suspension of EBV-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 2.5×10^4 CD3/kg recipient weight.

6.2.3. HLA-related mismatched donors (mismatch at 1-5 antigens/alleles) Dose 0.5×10^4 CD3/kg recipient weight.

6.2.4 Additional doses of EBV CTLs: (a minimum of every 2 weeks with a maximum of 5 infusions total)
6.2.4.1 - If recipients fail to respond to the first dose of EBV 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
6.2.4.2 Dose in HLA related matched donors: Max dose with each infusion 2.5 x 10^4 CD3/kg until a max combined dose of 12.5 x 10^4 CD3/kg (5 total doses)
6.2.4.3 Dose in HLA related Mismatched donors: Max dose with each infusion of 0.5 x 10^4 CD3/kg with a max combined dose of 2.5 x 10^4 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. 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-EBV agents and treatment with DLIs are prohibited until Week 12, and will be considered off study if new systemic anti-EBV therapy is initiated

6.4 Monitoring:

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

6.5 Hold EBV CTL infusion if any of the following present:

A) Thymoglobulin or alemtuzumab within 30 days B) uncontrolled infection or history of 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 EBV 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 EBV 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 EBV CTL infused.

H) Recipient septicemia is determined to be caused or potentially caused by contaminated related donor EBV CTL infusion

I) Performance Status of less than 30%.

If none of these criteria exist EBV 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 EBV CTLs

Patients with grade III-IV infusional toxicity probably or definitely attributable to related donor EBV CTL infusion 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.

7.2 Pre EBV-CTL Infusion Observations

7.2.1 History and physical examination: A complete history and physical examination including weight, height, BSA.
7.2.2 Hematology (must be within one week prior to starting therapy): WBC, differential, platelet count
7.2.3 Chemistry: Electrolytes, serum creatinine, BUN, total and direct bilirubin, SGPT(ALT), SGOT (AST), albumin, calcium, phosphorus, uric acid and magnesium, LDH.
7.2.4 Performance Status: Karnofsky or Lansky (age appropriate)
7.2.5 Baseline chimerism Study: on CTL donor and recipient
7.2.6 Plasma or Serum EBV qRT-PCR. Baseline CT or PET scans if clinically indicated.
7.2.7 HLA typing: HLA A and B by intermediate resolution; DRB1 by high resolution on donor and recipient
7.2.8 Urine pregnancy test for females of child bearing age
7.2.9 Characterization and Functional Assessment of the EBV CTL Clinical Grade Product (Appendix 4)

7.2.10 Characterization on Validations EBV CTL product (Appendix 3) (Minimum 2 validations per site)
7.2.11 Detection of EBV CTLs in the Blood of Patients Prior to Infusion of EBV CTLs (Appendix 5)
7.2.12 Pre-existing HLA Antibodies (Appendix 12)
7.2.13 All other laboratory monitoring according to the treating physician/standard of care

7.3 Post EBV CTL Infusions Observations

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

8.1 Manufacture

8.1.1. Manufacturing of the 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 EBV Select (containing among other antigens, NA-1, LMP2A and BZLF-1)). 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 EBV-specific T cells exceeds the number defined for the first dose of CTLs, the remaining 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 have the 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 EBV-specific T cells bags will be labeled in accordance with FDA applicable regulatory requirements.

8.1.4.2. Storage

The EBV-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 10 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.

The sample size is determined based on feasibility rather than statistical properties, given the rareness of the disease. Based on previous data, it is expected, that approximately 5% of patients will develop acute GVHD grade 3 and 4 probably or definitely related to related donor EBV CTLs. For cohort 1, with 10 patients and 1 observed Grade III/IV acute GVHD, the estimated Grade III/IV GVHD rate will be 10% with 95% exact confidence interval (CI) [0.2%, 45%], and if no Grade III/IV acute GVHD is observed then the estimated rate will be 0% with 95% exact CI [0%, 31%]. For the efficacy endpoint, we expect that approximately 25% of patients will obtain a complete response to EBV-CTLs with undetectable viral load by qRT-PCR by week 12. 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%]. 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 EBV CTL infusion i.e., when all patients have either completed Week 12 after EBV 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 EBV 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 probably or directly to related donor EBV-CTLs within 8 weeks after last EBV 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 EBV-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 EBV viral load, as measured by qPCR by Week 12 after first dose of EBV-CTLs (maximum response).

Frequency tabulations of the number and percentage of patients with decrease in EBV 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 EBV-CTLs

Complete Response: EBV-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 EBV 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 EBV 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 EBV-CTLs and if necessary at 45 days after the sixth patients is infused with EBV-CTLs (Section 9.3.4) with particular attention to Grade III-IV acute GVHD probably or directly related to EBV CTLs. These three patients have already been safely analyzed and will be included in the final 10cohort. 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 probably or definitely related to EBV-CTLs 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 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 probably or directly related to EBV-CTLs 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

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

9.4.2. Infusional Toxicity, Hematopoietic Graft Failure and Cytokine Release Syndrome Probably or Directly Related to EBV-CTLs

Infusional toxicity ≥ grade 3 (NCI CTCAE v 4.0), hematopoietic graft failure and CRS ≥grade 3 [33] probably or directly related to EBV-CTLs 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.

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

. 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 EBV 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 chemotherapy. 9.6.2An 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 EBV-CTLs infusion causing any one of the following complications:

9.6.2.1 Death,
9.6.2.2A 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 EBV-CTLs 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 CTL infusion, which upon investigation, is determined to be caused or potentially caused by the EBV-CTLs;

9.6.2.6.3 Recipient bacteremia secondary to contaminated EBV-CTLs.

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

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

9.6.3 Dose limiting criteria defined as:

9.6.3.1 Grade 3-5 infusion reaction, hematpoietic graft failures or CRS according to the NCI CTCAE 5.0 probably, or definitely related to EBV-CTL infusions

9.6.3.2 Hematopoietic graft failure, if applicable, is defined as patients following allogeneic stem cell transplantation with a neutrophil count $\leq$ 500/mm3 x 7 consecutive days with donor chimerism $\leq$ 20% after 60 days post allogeneic stem cell transplantation.

Any grade 3-5 adverse event considered probably or definitely related to the EBV-CTL 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 Co-Chairs, Drs. Nancy Bunin and Julie Talano, and Institutional Review Board reporting

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

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

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 EBV-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 EBV-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 EBV-CTL 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 EBV-CTL grade III or IV infusion toxicity

10.2 Progression of viral infection requiring new systemic anti-EBV 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 EBV-CTL infusion.

10.6 Lost to follow-up

V1 5.16.17 V3 2.1.18 V5 11.20.18 V7 11.1.19 V9 8.31.2020. V11 7.4.21
V2 8.16.17 V4 6.28.18 V6 6.3.19 V8 6.8.2020 V10 12.22.2020. V12 10.4.21
V13 1.3.2022. V14 4.27.22 V15 6.1.22. V15.1 8.22.22. V16 7.1.;23

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. Zutter MM, Martin PJ, Sale GE, et al: Epstein-Barr virus lymphoproliferation after bone marrow transplantation. Blood 72:520-9, 1988
12. Shapiro RS, McClain K, Frizzera G, et al: Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. Blood 71:1234-43, 1988
13. Styczynski J, Einsele H, Gil L, et al: Outcome of treatment of Epstein-Barr virus-related post-transplant lymphoproliferative disorder in hematopoietic stem cell recipients: a comprehensive review of reported cases. Transpl Infect Dis 11:383-92, 2009

14. 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

15. Cohen JM, Sebire NJ, Harvey J, et al: Successful treatment of lymphoproliferative disease complicating primary immunodeficiency/immunodysregulatory disorders with reduced-intensity allogeneic stem-cell transplantation. Blood 110:2209-14, 2007

16. 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

17. 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

18. 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

19. 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

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

21. 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

22. 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

23. 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

24. 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

25. 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

26. 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

27. 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

28. El Helou G, Rasonable RR: Safey considerations with current and emerging antiviral therapies for cytomegalovirus infection in transplantation. Expert Opin Drug Safe, 2019
29. 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
30. 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
31. 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
32. 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
33. Leen AM, Heslop HE, Brenner MK: Antiviral T-cell therapy. Immunol Rev 258:12-29, 2014
34. 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
35. 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
36. 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
37. 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
38. 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
39. Fahrendorff M vON, Rauser G et al: Automated generation of antigen-specific T cells for adoptive T cell therapy, 2010
40. 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
41. 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
42. 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

43. 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

44. 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

45. 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

46. 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

47. 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

48. 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

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

50. 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

51. Feucht J, Opherk K, Lang P, et al: Adoptive T-cell therapy with hexon-specific Th1 cells as a treatment of refractory adenovirus infection after HSCT. Blood 125:1986-94, 2015

52. 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

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

1.0 Principle: 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

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

3.2.2 Donor Questionnaire

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

3.2.3 Evaluation of response to Donor Questionnaire

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

3.3.2.1 Risk of RCDADs is identified

Donor is determined ineligible.

3.3.2.2 Other atypical response identified

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

The clinical program will refer to the the NMDP guidance documents and the evaluation tools listed in section **3.4** of the document to aid in decision making.

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)
- EBV- urine
- EBV 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.

3.5 Donor Confidentiality:

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

REFERENCES:

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

B. DONOR EVALUATION AND SCREENING FOR SUITABILITY

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

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

3.0 Procedure

3.1 Suitability – Applies to both autologous and 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.

V1 5.16.17 V3 2.1.18 V5 11.20.18 V7 11.1.19 V9 8.31.2020. V11 7.4.21
V2 8.16.17 V4 6.28.18 V6 6.3.19 V8 6.8.2020 V10 12.22.2020. V12 10.4.21
V13 1.3.2022. V14 4.27.22 V15 6.1.22. V15.1 8.22.22. V16 7.1.;23

2. Confer, DL. Stroncek, DF. Bone Marrow and Peripheral Blood Stem Cell Donors. In: Thomas Ed, Blume KG, Forman SJ (eds). Hematopoietic Cell Transplantation 2nd Ed. Malden, MA: Blackwell Science, Inc: 1999:421-430.

Appendix 2: Viral CTL Manufacturing

A. vCTL MANUFACTURING USING THE CliniMACS PRODIGY

1.0 Principle

1.1 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.

1.2 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.

1.3 The CliniMACS Cytokine Capture Cystem (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.

1.4 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.

2.0 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.

3.0 Specimen

3.1 T cells, Apheresis

3.2 T cells, Whole Blood

4.0 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 EBV select (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 EBV Consensus (6nmol)	Miltenyi
4.19 intracellular 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

5.0 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 CO_2 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

6.0 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

7.0 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 cryopreservation SOPs.

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 10^8/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 1x10^9 total nucleated cells for the manufacturing process on the Prodigy.

7.5.1 If the product is a whole blood, perform a RBC reduction using Ficoll per institutional RBC depletion SOPs. Since the expected recovery is at least 23%, a minimum of 4.44 x10^9 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 $1x10^9$ cells.

7.5.3 Final $1x10^9$ 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.

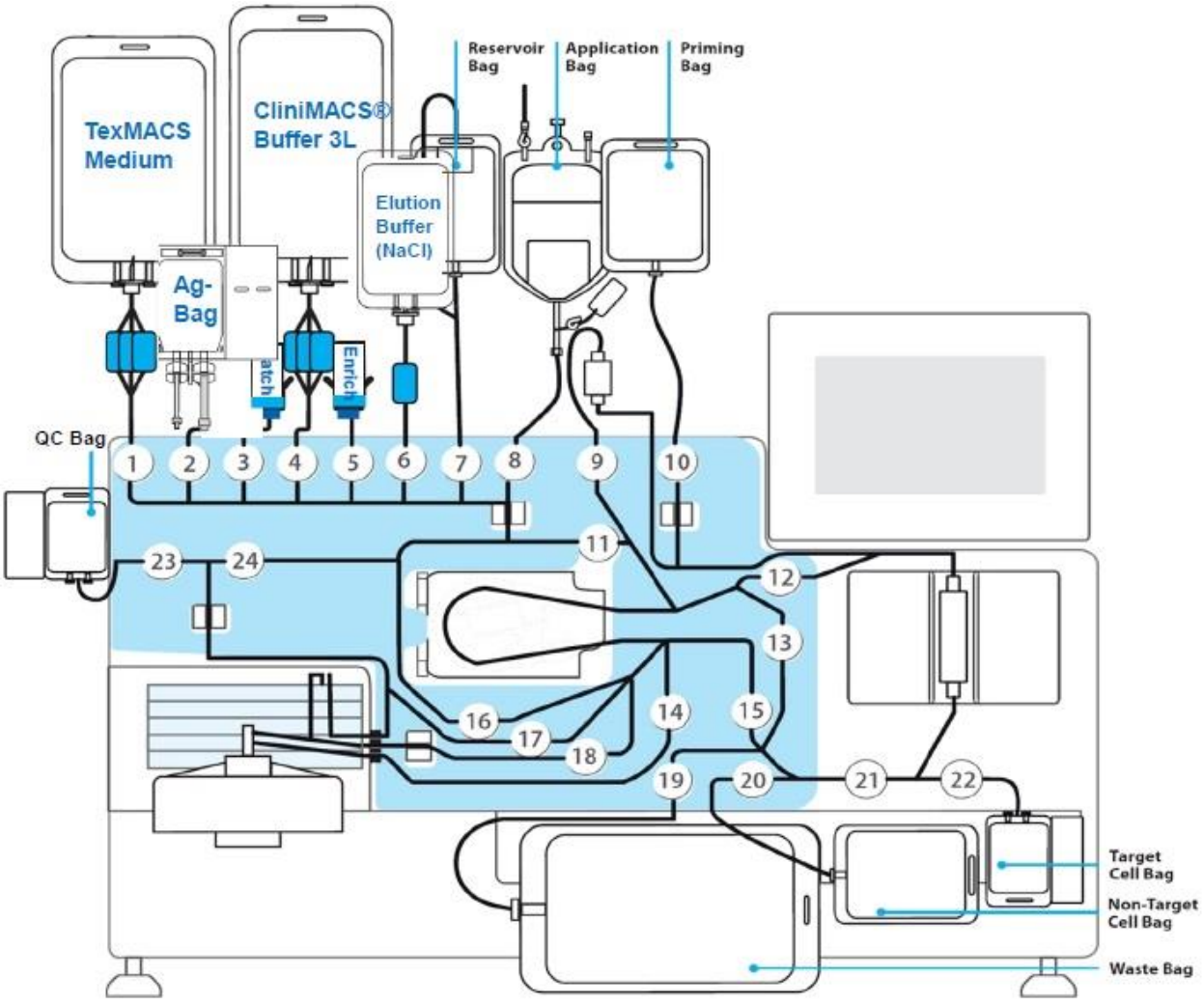
7.7.5 Tubing set must pass both the upper and lower part of integrity test.

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 $1x10^9$ 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 10^5-10^6 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 1-2x10^6 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 $5x10^6$ (total) cells/ml TexMACS at a density of $5x10e^6$ cells/cm^2, 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 ~1cm^2/well or ~2cm^2/well, respectively. Prepare a control well with NTC only at the same cell number. Culture in a 37C, 5% CO_2 incubator.

7.20.1 For example, $1x10^5$ TC is mixed with $1x10^7$ NTC, for a total of $1x10^7$ cells. These will be put in 2ml final volume and a 24well plate.

7.20.2 For example, $5x10^4$ TC is mixed with $5x10^6$ NTC, for a total of $5x10^6$ 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.

8.0 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

V1 5.16.17 V3 2.1.18 V5 11.20.18 V7 11.1.19 V9 8.31.2020. V11 7.4.21
V2 8.16.17 V4 6.28.18 V6 6.3.19 V8 6.8.2020 V10 12.22.2020. V12 10.4.21
V13 1.3.2022. V14 4.27.22 V15 6.1.22. V15.1 8.22.22. V16 7.1.;23

10.1 CliniMACS Prodigy CCS System User Manual. Miltenyi Biotec.

B. Donor PreScreening for Virus Specific Cytotoxic T Lymphocytes

1.0 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 staining measured by cell surface capture using a catch reagent, followed by flow cytometric analysis.

2.0 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.

3.0 Specimen

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

4.0 Supplies and reagents	Source
4.1 PepTivator® EBV consensus (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

5.0 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 CO_2 incubator

6.0 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

7.0 Procedure

7.1 Reconstitute the 6 nmol viral peptides (EBV) 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 EBV. 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.

7.2 The positive control used is 20ng/ml PMA and 1µg/ml ionomycin.

7.2.1 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.

7.2.2 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.

7.2.3 All aliquots used in steps 7.2 are for single use.

7.3 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 1-2x10^8 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.

7.4 Cells that have been refrigerated/frozen:

7.4.1 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 < 1x10^7/ml, and let the cells recover in a 37C incubator overnight. Non-viable cells and debris are expected.

7.4.1.1 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.

7.4.2 If using fresh cells that arrived in chilled state during transport/storage, culture them at 37C in TexMACS at < $1x10^7$/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.)

7.5 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 $2x10^8$ starting cells and a hypothetical 30% recovery, this gives $3x10^7$/ml x 2ml = $6x10^7$ cells.) Adjust to a final live cell concentration of ~$1x10^7$/ml. (Take into account trypan blue viability if using thawed cells).

7.6 Add 1ml (~$1x10^7$ 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% CO_2 for 4 hours.

7.6.1 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.

7.6.2 This step and later ones, if needed, can be performed on an open bench.

7.7 **COLD**. After 4 hours, mix the cells well and transfer 0.1ml (~$1x10^6$ 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.

7.8 **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.

7.9 **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 ~$1x10^6$ 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.

7.10 **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

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

8.0 Expected Results

8.1 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

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.

9.2 CD4/IFN□ and CD8/IFN□, determination by Flow Cytometry is performed on the post-stimulation product.

10.0 References

10.1 Rapid Cytokine Inspector kit. Miltenyi Biotec.

10.2 IFN□ secretion assay detection kit. Miltenyi Biotec

10.3 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)

2.0 Methods for Preparation of Cells

a. Each new center will perform 3 validations of either combination BKV, CMV, ADV or EBV CTLs.
b. 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

3.0 Shipping Instructions

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

Analysis	Recipient	Pre-stimulated PBMC	QC Sample	Target Cells	Priority	Shipping instruction
i. Single cell RNAseq analysis	Nationwide Children'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 -80°C 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 -80°C 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

4.0 Summary

5.0 Detailed Shipping Address

i. 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

ii. 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

iii. 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

iv. 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

v. 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

vi. Children's Hospital of Pennsylvania
Vella Lab
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 EBV CTL Product

1.0 Objective:

To characterize ex vivo expanded EBV CTL product (IFG+ cells from CCS product)

2.0 Methods for Preparation of Cells

c. From the CCS viral CTL target cell fraction, remove 0.5-1e5 cells (50-100k cells), wash once by centrifugation, resuspend in 1 ml TexMACS supplemented with 100 IU/ml IL-2, and place in a 1 ml cryovial. Tightly seal the cryovial in preparation for **immediate overnight shipping** to the Johnson Lab at MCW (at 4 °C).
d. From the CCS CTL isolation non-target cell fraction, concentrate and wash the cells once by centrifugation, and resuspend in 15 ml TexMACS. Place the cell suspension in a 15 ml screw-cap conical centrifuge, and tighten the cap in preparation for **immediate overnight shipping** to the Johnson Lab at MCW (at 4 °C). These cells will be used to manufacture BLCL lines and as feeders to expand an aliquot of the target cell fraction in the Johnson Lab.

3.0 Shipping Instructions

i. All samples should be securely packaged in a container designed for shipping human biospecimens.
j. 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.
k. 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

4.0 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

V1 5.16.17 V3 2.1.18 V5 11.20.18 V7 11.1.19 V9 8.31.2020. V11 7.4.21
V2 8.16.17 V4 6.28.18 V6 6.3.19 V8 6.8.2020 V10 12.22.2020. V12 10.4.21
V13 1.3.2022. V14 4.27.22 V15 6.1.22. V15.1 8.22.22. V16 7.1.;23

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

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

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

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

5.0 Objectives

- Determine whether detectable virus-specific T cells are present in the blood of CTL recipients prior to infusion of the CTL product

6.0 Methods for Preparation of Cells

a. 15 ml of whole blood should be collected in a green top tube (sodium heparin) from the CTL **recipient** just prior to infusion of the CTL product.
b. Please label the tube as "Pre-Infusion Sample", along with the other information listed below.
c. The blood sample should be shipped overnight at room temperature to the Johnson Lab at MCW.

7.0 Shipping Instructions

l. All samples should be securely packaged in a container designed for shipping human biospecimens.
m. The **whole blood sample** (green top tube) should be shipped overnight at room temperature.
n. 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

8.0 Shipping Address

BMT Research Laboratory
Attention: Huiqing Xu, MD
Froedtert Hospital Pavilion, Room 304
9200 West Wisconsin Avenue
Milwaukee, WI 53226
Laboratory telephone: 414-805-6143

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

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

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

9.0 Objective

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

10.0 Methods for Preparation of Cells

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

11.0 Timing of Sample Collection

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

12.0 Shipping Instructions

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

13.0 Shipping Address

V1 5.16.17 V3 2.1.18 V5 11.20.18 V7 11.1.19 V9 8.31.2020. V11 7.4.21
V2 8.16.17 V4 6.28.18 V6 6.3.19 V8 6.8.2020 V10 12.22.2020. V12 10.4.21
V13 1.3.2022. V14 4.27.22 V15 6.1.22. V15.1 8.22.22. V16 7.1.;23

Yaya Chu, PhD
Basic Science Building, Rm401
New York Medical College
40 Sunshine Cottage Road
Valhalla, NY, 10595
email: yaya_chu@nymc.edu. Lab 914-594-3726

Ship overnight express on the day of collection;
Specimens will only be received Tuesday through Friday (except Holidays).

Appendix 7: Acute GVHD Grading (CIMBTR)

Acute GVHD: Clinical Stage

	Skin	Liver	Gut
Stage	% BSA	Bilirubin (mg/dl)	Diarrhea (ml/day)
I	<25	2-3	500-1000
II	25-50	3.1-6	1000-15000
III	Generalized erythroderma	6.1-15	>1500
IV	Bullae	>15	Pain+/-ileus

Acute GVHD: Clinical Grade

Overall Grade	Skin	Liver	GI	Upper GI
1	1-2	0	0	0
II	1-3	1	1	1
III	2-3	2-4	2-3	
IV	4	-	4	

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

	Screening	T-cell transfer	FU I	FU II						
	Visit I	II	III	IV	V	VI	VII	VIII	XI	X
	Day –21	Day 0	Day 1	Day 7	Week 2 (±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	X[a]		X[a]	X[a]	X[a]	X[a]	X[a]	X	X
Hematology[a]	X		X	X	X	X	X	X		
Chemistry	X									
EBV qRT-PCR	X,	X		X[a]	X[a]	X[a]	X[a]	X[a]		
T-cell Chimerism in third-party donors	X				X					
Immune Studies	X	X					X	X	X	X
Physical examination	X									
Vital signs	X									
Performance Status	X					X	X	X	X	X
AEs/SAEs		X	X	X	X	X	X	X	X	X
Concomitant anti-viral medication	X	X	X	X	X	X	X	X		
CTL Biology (Appendix III, IV, V, VI)	X	X			X		X	X		

a: weekly for 12 weeks post CTL infusion

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

CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Infusion related reaction	Mild transient reaction; infusion interruption not indicated; intervention not indicated	Therapy or infusion interruption indicated but responds promptly to symptomatic treatment (e.g., antihistamines, NSAIDS, narcotics, IV fluids); prophylactic medications indicated for <=24 hrs	Prolonged (e.g., not rapidly responsive to symptomatic medication and/or brief interruption of infusion); recurrence of symptoms following initial improvement; hospitalization indicated for clinical sequelae	Life-threatening consequences; urgent intervention indicated	Death
Definition: A disorder characterized by adverse reaction to the infusion of pharmacological or biological substances.					

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

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

Reproduced from: Common Terminology Criteria for Adverse Events (CTCAE), Version 5.0, November 2017, National Institutes of Health, National Cancer Institute. Available at: https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_8.5x11.pdf

Appendix 10 : Cytokine Release Syndrome Grading System*

Grade	Toxicity
Grade1	Symptoms are not life threatening and require symptomatic treatment only, eg. Fever, nausea, fatigue, headache, myalgia's, 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 respond 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 11: Severe Chronic GVHD

Mild chronic GVHD

1 or 2 organs involved with no more than score 1 *plus*
Lung score 0

Moderate chronic GVHD

3 or more organs involved with no more than score 1

OR

At least 1 organ (not lung) with a score of 2

OR

Lung score 1

Severe chronic GVHD

At least 1 organ with a score of 3

OR

Lung score of 2 or 3

Key points:

1. In skin: higher of the two scores to be used for calculating global severity.
2. In lung: FEV1 is used instead of clinical score for calculating global severity.
3. If the entire abnormality in an organ is noted to be unequivocally explained by a non-GVHD documented cause, that organ is not included for calculation of the global severity.
4. If the abnormality in an organ is attributed to multifactorial causes (GVHD plus other causes) the scored organ will be used for calculation of the global severity regardless of the contributing causes (no downgrading of organ severity score).

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

APPENDIX 12: Pre-existing HLA Antibodies study

1.0 Objective

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

2.0 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.

3.0 Timing of Sample Collection

- Prior to the first viral CTL infusion to patient.

4.0 Shipping Instructions

r. All samples should be securely packaged in a container designed for shipping human biospecimens.
s. The isolated serum should be shipped overnight with dry ice.
t. 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

5.0 Shipping Address

Yaya Chu, PhD
Basic Science Building, Rm401
New York Medical College
15 Dana road
Valhalla, NY, 10595

V1 5.16.17 V3 2.1.18 V5 11.20.18 V7 11.1.19 V9 8.31.2020. V11 7.4.21
V2 8.16.17 V4 6.28.18 V6 6.3.19 V8 6.8.2020 V10 12.22.2020. V12 10.4.21
V13 1.3.2022. V14 4.27.22 V15 6.1.22. V15.1 8.22.22. V16 7.1.;23

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.