SOP: D03.32.21 EXPANSION OF EBV- OR ANTIGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTES (CTL)

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1. Purpose

1.1. This SOP is a part of a series of procedures involved in the manufacture of Cytotoxic T cell Lymphocytes (CTL).

1.2. The worksheets contain expansion procedures involved in generating sufficient cells for the patient dose requirement, QC testing and research.

1.3. Worksheets are written for the expansion of the following:

1.3.1. Epstein Barr Virus-specific cytotoxic T cell lines (CTL) generated by stimulation of peripheral blood mononuclear cells (PBMC) with autologous (EBV) transformed B cell lines (LCL).

1.3.2. Antigen-specific cytotoxic T cell lines (CTL) generated by stimulation of peripheral blood mononuclear cells (PBMC) with autologous antigen-presenting cells (APC) expressing the antigen from an adenovirus vector.

1.3.2.1. The antigen presenting cells used for expansion are Epstein-Barr virus (EBV)-transformed B cell lines (LCL) that may be transduced with ad adenovirus vector containing a transgene using SOPs D03.01 and D03.14.

1.4. Under the culture conditions employed, outgrowing T cell lines should contain T cells specific for the antigen(s) or virus of interest.

1.5. Additional cytokines (IL-15, IL-4, IL-7 and IL-12) may be added at the first or second stimulation depending on the Protocol.

2. Scope

2.1. This procedure is to be followed by trained GMP staff when manufacturing EBV- or Antigen-specific cytotoxic T lymphocytes.

2.2. Current and future Protocols that will use this SOP. Listed are some of the current Protocols that will use this SOP are listed below.

ALCI:	Ad5f35LMP1-I-LMP2 –specific T cells
Natella:	Ad5f35LMP1-I-LMP2 –specific T cells
TGFbeta:	Ad5f35LMP1-I-LMP2 –specific T cells transduced with SFG.TGFβRIIδcyt (DNRII) vector

GBM/HER2: EBV-specific CTL transduced with retrovirus vectors expressing HER2-CAR and a dominant-negative TGF–β receptor.

ATECRAB: EBV-specific CTL transduced with a retrovirus vector expressing CD19-CAR

MULTIPRAT: Viral antigen-specific CTL transduced with a retrovirus vector expressing CD19-CAR

3. Definitions and Abbreviations

3.1.	CTL	Cytotoxic T- lymphocytes
3.2.	Transgene	Gene expressed from a viral vector to stimulate T cells
3.3.	Gy	Gray - unit of radiation
3.4.	PBMC	Peripheral blood mononuclear cells
3.5.	DC	Dendritic Cells
3.6.	EBV	Epstein Barr Virus
3.7.	LCL	Lymphoblastoid Cell Line
3.8.	HI	Heat Inactivated
3.9.	D-PBS	Dulbecco's Phosphate Buffered Saline
3.10.	i.u.	Infectious Units
3.11.	FCS	Fetal Calf Serum
3.12.	CTL medium	45%RPMI 1640 or Advanced RPMI, 45%EHAA (Click's) containing 10% HI FCS and 2mM L-glutamine
3.13.	QC	Quality Control
3.14.	BSC	Biological Safety Cabinet

4. Materials and Equipment

NOTE Materials in contact with cells must be sterile, pyrogen-free, stored and used according to the manufacturer's directions unless stored otherwise. Equivalent materials and equipment may be used but all changes must be approved by Quality Assurance

4.1. Specimens

4.1.1. EBV or antigen-specific CTL from D03.31

4.1.2. Transduced EBV or antigen-specific CTL from D03.33

4.1.3. Lymphoblastoid Cell Line (LCL) prepared from the patient or donor and cultured for > 2 weeks in 100 µM Acyclovir

4.1.3.1. Unmodified from D03.01 or

4.1.3.2. Transduced with adenovirus vector (Ad5f35ΔLMP1-I-LMP2) or Ad5f35-pp65 or Ad5f35-IE1-I-pp65 according to SOP D03.14.

4.2. Materials

4.2.1.	RPMI 1640	Invitrogen
4.2.2.	Advanced RPMI 1640	Invitrogen
4.2.3.	EHAA (Click's Medium)	Irvine Scientific
4.2.4.	HI Fetal Calf Serum	HyClone
4.2.5.	L-glutamine (200 mM)	Invitrogen
4.2.6.	Tissue culture plates	Costar
4.2.7.	GRex flasks	Wilson Wolf
4.2.8.	Interleukin-2 (Proleukin)	Chiron (TCH Pharmacy)
4.2.9.	Centrifuge tubes	Falcon
4.2.10.	Serological Pipets	Falcon
4.2.11.	Pipette tips	VWR
4.2.12.	Lymphoprep	Nycomed
4.2.13.	Dulbecco's PBS	Invitrogen
4.2.14.	Trypan Blue	

4.3. Equipment

4.3.1.	Biological safety cabinet (BSC), certified	
4.3.2.	Microscope	Olympus

4.3.3. Centrifuge

4.3.4. Incubator

4.3.5. Irradiator

4.3.6. Hemacytometer

4.3.7. Water bath

4.3.8. Pipetman

4.3.9. Pipet Aid, automatic pipettor

4.4 Worksheets

4.4.1 DW03.32.xxA Expansion of EBV or antigen-specific CTL in 24 well plates

4.4.2 DW03.32.xxB Expansion of EBV or antigen-specific CTL in GRex flasks

5. Expansion of EBV or antigen-specific CTL:

NOTE 2: The Principal Investigator should have calculated the final expanded T cell numbers required in D03.31

5.1 Harvest stimulated CTLs from D03.31 (Initiation of CTLs) or D03.33 (Transduction of CTLs) and restimulate:

5.1.1 In 24 well plates for the 3rd or subsequent stimulation according to 5.4 and 5.5

5.1.2 In GRex flasks for the 2nd or subsequent stimulation according to 5.6-5.10

5.2 Cryopreserve excess T cells as back up if sufficient (use SOP D03.21 – worksheet DW03.21.xxA for In Process Cryopreservation of Suspension Cells).

5.3 Super-expand if cell growth is poor proceed to SOP D03.03

5.3.1 For example if the cell numbers have not at least doubled in one week)

5.3.2 Transduce after the 4th stimulation if super-expanded at the 3rd stimulation.

5.4 **Third or subsequent stimulation in 24 well plates**

5.4.1 *Harvest CTLs from D03.31 or D03.33*

5.4.2 Count and determine viability

5.4.2.1 If viability is > 60% proceed to section 5.4.3

5.4.2.2 If viability is less than 60%, contact PI

5.4.2.2.1 Centrifuge over Lymphoprep gradients to remove dead cells

5.4.2.2.2 Harvest interface, centrifuge and re-suspend in medium. Count cells and wash one more time.

5.4.3 Resuspend CTLs at 1 x 10^6 cells per mL CTL medium

5.4.3.1 Add 100 to 200 units per mL of IL-2 (50 to 100 units/ml final dilution) - 0.5 to 1.0 μL of IL-2 per mL CTLs using IL-2 stock (200 units per μL)

5.4.4 Aliquot 1ml cells per well of a 24 well plate

5.4.5 *Restimulate with autologous LCL*

5.4.5.1 Unmodified LCL from D03.01

5.4.5.2 Record removal on LCL worksheet DW03.01.XX

5.4.5.3 Irradiate 40 Gy, wash and count

5.4.6 LCL transduced with an adenovirus vector

5.4.6.1 Irradiated, washed 4 times and resuspended at 2.5x 0^5 cells per mL according to SOP D03.14 - may be fresh (DW03.14.xxA) or cryopreserved (DW03.14.xxB)

5.4.6.2 Record LCL removal on LCL worksheet and record LCL production report number on CTL worksheet

5.4.7 Re-suspend LCL at 2.5 x 10^5 cells per mL and aliquot 1 ml per well of responder T cells

5.4.8 Culture 2mLs of irradiated LCL alone in one well as a control for irradiation

5.4.9 Culture for 3-4 days at 37^o C in 5% CO2 in air.

5.5 **IL-2 feed in 24 well plates**

5.5.1 Remove ~ 1 mL medium from each well of CTLs

5.5.2 Replace with ~ 1 mL medium containing 100 to 200 units per mL of IL-2 (50 to 100 units final dilution) - 0.5 to 1.0 μL of IL-2 per mL from IL-2 stock (200 units per μL)

5.5.3 Return to culture for 3 to 4 days

5.5.4 Repeat 5.4 and 5.5 until sufficient cells for the protocol have been obtained, then cryopreserve for infusion according to D03.05

5.6 **Second or subsequent CTL stimulation in a GRex flask at a 5:1 ratio of LCLs : CTLs**

Note: CTLs can be expanded in bioreactor for up to 16 to 18 days.

Note: CTLs can be cryopreserved for infusion from day 7, but immunologic analysis should not be performed until day 10 or later because of a high background cytokine release

Note: The second stimulation is without IL-2 (IL-2 is added 3-4 days later). The third and subsequent stimulations use IL-2

5.6.1 Harvest responder cells from 24-well plates or GRex40 bioreactor.

5.6.2 Count and determine viability

5.6.3 If viability is > 60% proceed to section **5.6.5**

5.6.4 If viability is less than 60%, contact PI

5.6.4.1 Centrifuge over Lymphoprep gradients to remove dead cells

5.6.4.2 Harvest interface, centrifuge and re-suspend in medium. Count cells and wash one more time.

5.6.5 Re-suspend CTL at 1 x 10^6 cells per mL CTL medium

5.6.6 Transfer 1 to 5ml CTL into GP40 or 10 mLs into GP500

5.6.7 Restimulate with autologous LCLs at a 5:1 ratio of LCLs:CTLs

5.6.7.1 Unmodified LCL from D03.01

5.6.7.1.1 Record removal on LCL worksheet DW03.01.XX

5.6.7.1.2 Record LCL production report # on CTL worksheet

5.6.7.1.3 Irradiate 80 Gy, wash and count

5.6.8 LCL transduced with an adenovirus vector

5.6.8.1 Irradiated at 80 Gy, washed 4 times according to SOP D03.14

5.6.8.2 May be fresh (DW03.14.xxA) or cryopreserved (DW03.14.xxB)

5.6.8.3 Record LCL removal LCL worksheet and record LCL production report number on CTL worksheet

5.6.9 Transfer 5 x 10^6 LCLs to GRex40 or 5 x 10^7 LCLs to GRex500

5.6.10 Adjust GRex40 to 30 mLs or GRex500 to 200 mLs with medium

5.6.11 Add 50 to 100 units per mL of IL-2 (7.5 to 15 μL in GRex40 or 50 to 100 μL inGRex500)

5.6.12 Culture for 3-4 days at 37°C in 5% CO2 in air.

5.6.13 Culture LCLs alone to control for irradiation in small flask or well

5.7 **First IL-2 feed in the GRex**

5.7.1 Remove 20 mL medium from GRex40

5.7.2 Prepare IL-2 medium for a final concentration of 50 to 100 units per mL

5.7.2.1 Using 200 units per μL stock IL-2

5.7.2.2 Add 20 mL medium containing 7.5 to 15 μL IL-2 to GRex40

5.7.2.3 Add 200 mL medium containing 100 to 200 μL of IL-2 to GRex500

5.7.3 If planning to cryopreserve in 3 to 4 days, notify ancillary staff - PI, QC and Flow cytometry

5.7.4 Culture for 3 to 4 days

5.8 **Second IL-2 feed; expand in the GRex or cryopreserve**

5.8.1 Remove ~ half medium from each GRex

5.8.1.1 20 mL from GRex40

5.8.1.2 200 mL from GRex500

5.8.2 Resuspend cells in remaining medium and count

5.8.3 If sufficient cells for infusion and QC, cryopreserve using D03.05

NOTE: Cells for immunological analysis must be cultured for 3 to 4 more days in low dose IL-2 before analysis in GMP or on research floor, immediately or after cryopreservation

5.8.4 If insufficient cells for infusion

5.8.4.1 If greater than 50 x 10^6 cells in GRex40 or greater than 500 x 10^6 cells in GRex500 spilt 1:1 into identical GRex(s)

5.8.4.2 Excess cells can be frozen as back up if two flasks are not needed

5.8.4.3 If less than 50 x 10^6 cells in GRex40 or less than 500 x 10^6 cells in GRex500 feed with IL-2 medium

5.8.5 Prepare IL-2 medium for final concentration of 50 to 100 units per mL

5.8.5.1 20 mL for GRex40(s) (7.5 to 15 μL IL-2)

5.8.5.2 200 mL for GRex500(s) (100 to 200 μL IL-2)

5.8.6 If planning to cryopreserve in 3 to 4 days, notify ancillary staff - PI, QC and Flow cytometry

5.8.7 Culture for 3 to 4 days

5.9 **Third feed; expand in the GRex or cryopreserve**

5.9.1 Remove half medium from each GRex

5.9.1.1 20 mL from GRex40

5.9.1.2 200 mL from GRex500

5.9.2 Resuspend cells in remaining medium and count

5.9.3 If sufficient cells for the patient doses, cryopreserve according to D03.05

5.9.4 If insufficient cells

5.9.4.1 If greater than 50 x 10^6 cells in GRex40 or greater than 500 x 10^6 cells in GRex500 spilt 1:1 into identical GRex(s)

5.9.5 Backup freeze excess cells if not required

5.9.6 Move to 5.9.8

5.9.7 If less than 50 x 10^6 cells in GRex40 or less than 500 x 10^6 cells in GRex500 feed with IL-2 medium (5.9.8)

5.9.8 Prepare IL-2 medium for final concentration of 50 to 100 units per mL

5.9.8.1 20 or 25 mL medium (final 30 mL) for GRex40(s) (7.5 to 15 μL of IL-2)

5.9.8.2 200 mL medium for GRex500(s) (100 to 200 μL IL-2)

5.9.9 If planning to cryopreserve in 3 to 4 days, notify ancillary staff - PI, QC and Flow cytometry

5.9.10 Culture for 3 to 4 days

5.10 **Cryopreserve or restimulate**

5.10.1 Remove half medium from each GRex

5.10.1.1 20 mL from GRex40

5.10.1.2 200 mL from GRex500

5.10.2 Resuspend cells in remaining medium and count

5.10.3 If sufficient cells for the patient doses, cryopreserve according to D03.05

5.10.4 If insufficient cells for the patient dose restimulate with LCL (repeat this SOP D03.32)

5.10.4.1 Superexpand if necessary using D03.03

NOTE: Perform all steps in a certified biological safety cabinet, using aseptic technique.

7. Notes

7.1 Since all cells prepared are intended for infusion to patients, it is essential to adhere to proper procedures to prevent misidentification or contamination of patient samples.

7.2 ALL culture vessels and centrifuge tubes must be labeled with information to include patient name, patient number and patient's date of birth. **UNLABELED MATERIAL WILL BE DISCARDED.**

7.3 **NEVER** work with more than one patient cell line at any one time.

7.4 **ALWAYS** use medium prepared and labeled specifically for each patient's cells. NEVER use medium to feed more than one patient's cells.

7.5 Perform all steps in a certified biological safety cabinet, using aseptic technique and following universal precautions.

8. Expected Results

8.1 The cells should have the following characteristics:

8.1.1 Sufficient CTL numbers for infusion of the patient at the appropriate dose level (determined at that time) and for all QC requirements.

8.1.2 < 10% killing of recipient PHA blasts at 20:1 (if allogeneic)

8.1.3 <2% CD19+ B cells by flow cytometry (exclusion of LCL)

8.1.4 <2% CD 83+/$CD3^-$ cells (exclusion of DC) if appropriate

8.1.5 <2% CD14+ cells (exclusion of monocytes) if appropriate

8.1.6 Expression of transgene according to protocol

9. Worksheets

9.1 DW03.32.xxA Generation of EBV or antigen-specific CTLs in a 24-well plate

9.2 DW03.32.xxB Generation of EBV or antigen-specific CTLs in a GRex flask

10. References:

10.1 Rooney CM, Smith CA, Ng CYC, Loftin SK, Sixbey JW, Gan YJ, Bowman LC, Krance RA, Brenner MK, Heslop HE. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. Blood. 1998 Sep 1;92(5):1549-55.

10.2 Leen AM, Myers GD, Sili U, Huls MH, Weiss H, Leung KS, Carrum G, Krance RA, Chang C-C, Molldrem JJ, Gee AP, Brenner MK, Heslop HE, Rooney CM, Bollard CM. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. Nat Med. 2006 Oct;12(10):1160-1166.

10.3 Leen AM, Ratnayake M et al. Contact-activated monocytes: efficient antigen presenting cells for the stimulation of antigen-specific T cells. J Immunother. 2007 Jan;30(1):96-107

10.4 Bollard CM, Gottschalk S, Leen AM, Weiss H, Straathof KC, Carrum G, Khalil M, Wu MF, Huls MH, Chang CC, Gresik MV, Gee AP, Brenner MK, Rooney CM, Heslop HE. Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocytes transfer. Blood 2007 Oct 15:110(8):2838-45.

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11. Review and Revisions:

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