SOP: D03.31.22 INITIATION OF EBV- OR ANTIGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTES (CTL)

If using a printed copy, insert print date here :_____(on first page only)

1. Purpose

1.1. This SOP contains worksheets specific for initiating cytotoxic T cell lines (CTL).

1.2. The worksheets contain procedures starting from initiation by stimulation and second stimulation with APCs far as the first expansion with IL-2 according to SOP D03.31

1.3. Worksheets are written for the following:

1.3.1. Epstein Bar Virus (EBV)-specific cytotoxic T cell lines (CTL) initiated 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.

1.3.3. The antigen presenting cells may be dendritic cells (DC) or adherent monocytes for the first stimulation and Epstein-Barr virus (EBV)-transformed B cell lines (LCL) for the second and subsequent stimulations.

1.4. Under the culture conditions employed, outgrowing T cell lines should contain T cells specific for the antigen 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.

2.2.1. ETNA, ALCI, TGF–β, ATECRAB, CHALLAH, NATELLA, HERCREEM, HEROS, NESTLES, VICTA, HERT-GBM, COGLI

2.3. Each protocol will have its own SOP that will list the APCs and responder cells used.

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.	Monocyte medium	X-Vivo 15 with 2mM L-glutamine
3.14.	QC	Quality Control
3.15.	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. Heparinized peripheral blood from the patient or donor, or previously frozen patient or donor PBMC processed according to D03.30

4.1.2. Infectious disease testing must be performed within 7 days (depending on specific protocol) of blood collection.

4.1.2.1. Cryopreserved PBMC should be stored in quarantine results are obtained, then transferred out of quarantine if results are appropriate

4.1.3. DC prepared from the patient or donor and transduced with an adenovirus vector according to SOP D03.15.

4.1.3.1. The adenovirus vector is specified by the protocol-specific SOP

4.1.4. Lymphoblastoid Cell Line (LCLs) prepared from the patient or donor according to SOP D03.01 cultured for > 2 weeks in 100 µM acyclovir

4.1.5. LCL transduced with adenovirus vectors according to SOP D03.14

4.1.6. Adenovirus vector – Ad5f35ΔLMP1-I-LMP2

4.1.7. Adenovirus vector – Ad5f35CMVpp65

4.2. Materials

4.2.1.	RPMI 1640	Invitrogen
4.2.2.	Advanced RPMI 1640	Invitrogen
4.2.3.	X-vivo 15	Lonza Walkersville, Inc
4.2.4.	EHAA (Click's Medium)	Irvine Scientific
4.2.5.	HI Fetal Calf Serum	HyClone
4.2.6.	L-glutamine (200 mM)	Invitrogen
4.2.7.	IL-15	CellGenix
4.2.8.	Tissue culture plates	Costar
4.2.9.	Bioreactors (GRex)	Wilson Wolf
4.2.10.	Interleukin-2 (Proleukin)	Chiron (TCH Pharmacy)
4.2.11.	Centrifuge tubes	Falcon
4.2.12.	Serological Pipets	Falcon
4.2.13.	Pipette tips	VWR
4.2.14.	Lymphoprep	Nycomed
4.2.15.	Dulbecco's PBS	Invitrogen
4.2.16.	Red Cell Lysis Buffer	Becton Dickinson

4.2.17. 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 DW03.31.xxA Initiation of EBV-specific CTL in plates or GRex

4.4.2 DW03.31.xxB Initiation of Antigen-specific CTL using Monocytes

4.4.3 DW03.31.xxC Initiation of Antigen-specific CTL using Adenoviral vector-transduced dendritic Cells (DC)

5. Procedure – Initiation and expansion of EBV- or antigen-specific CTL

NOTE 1: If the CTL are produced from an allogeneic donor, please ensure that RECIPIENT blood has been collected and submitted to the GLP laboratories for production of PHA blasts. If insufficient RECIPIENT blood is available - an additional blood sample should be requested by the Principal Investigator or research nurse associated with the study.

NOTE 2: Autologous LCLs should have been established and grown in acyclovir for at least two weeks using SOP D03.01

5.1 The Principal Investigator should calculate the final expanded T cell numbers required.

5.1.1 Include patient doses according to the patient's body surface area, predicted dose levels and whether additional doses are allowed.

5.1.2 If possible allow for the chance that the patient may be enrolled on a higher dose level than expected.

5.1.3 Include cells for QC and for research

Table 1 Sample Dose calculation

Expected dose level	x BSA	=	+20% (for loss)	= Cell number
2×10^7	2	4×10^7	0.8×10^7	4.8×10^7
			+ 1.5×10^7 for QC	6.3×10^7
			+ 2×10^7 for research **(subtotal)**	**8.3×10^7**
For retrovirus vector-transduced CTLs add 3% of final product but not less than 1.5×10^7 (1.5×10^7)				**9.8×10^7**
Minimal number of transduced CTLs to be grown is				**9.8×10^7**

5.2 **Preparation of mononuclear "responder" cells from fresh blood (for frozen blood, proceed to step 5.3)**

5.2.1 Dilute heparinized peripheral blood (ideally 30ml) in an equal volume of D-PBS or RPMI 1640 at ambient (room) temperature.

5.2.2 In a 50 ml centrifuge tube, carefully overlay approximately 10ml Lymphoprep with approximately 20 ml of diluted blood.

5.2.3 Adjust as necessary to utilize all the available cells.

5.2.4 Centrifuge at 400 x G for 40 minutes at ambient temperature.

5.2.5 Save 3 x 1 ml plasma aliquots and store at -80^{o} C.

5.2.6 Harvest PBMC interface into an equal volume of D-PBS or RPMI 1640.

5.2.7 Centrifuge at 450 x G for 10 minutes at room temperature. Aspirate supernatant.

5.2.8 Loosen pellet by “finger-flicking” and resuspend in 20ml of D-PBS or RPMI 1640.

5.2.9 Remove 20µl of cells. add 20µl of 50% red cell lysis buffer and count using a hemacytometer according to SOP H05.02 (see 5.1.1).

5.3 Preparation of responder cells from previously frozen PBMCs

5.3.1 Thaw cells at 37^{o}C, dilute in 9 mL of warm medium per 1 mL of frozen cells.

5.3.2 Count according to SOP HO5.02

5.3.3 Obtain mononuclear "responder" cells from SOP D03.20. - May be fresh or frozen PBMC or non-adherent mononuclear cells

5.4 EBV-specific CTL initiation (PBMC stimulation by lymphoblastoid cells (LCL) at 40:1 ratio of PBMC to LCL)

5.4.1 Centrifuge PBMC at 400G for 5 minutes at room temperature.

5.4.2 Remove supernatant and re-suspend at $2x10^6$ cells per mL.

5.4.3 Aliquot 1mL of cells ($2x10^6$) per well of a 24-well plate or

5.4.4 Aliquot 5mL ($1x10^7$) into GRex40 bioreactor

5.4.5 Transfer plates or GRex40 to incubator.

5.5 Obtain LCL from SOP D03.01

5.5.1 Note volume of LCL removed on DW03.01.

5.5.2 Note LCL production report#

5.5.3 Irradiate with 40GY.

5.5.4 Count using SOP H05.02, then centrifuge at 400xG for 5minutes.

5.5.5 Re-suspend LCL at $5x10^4$ cells per mL.

5.5.6 Transfer PBMC in 24well-plates or GRex40 at a ratio of 40:1 of PBMC to LCL.

5.5.6.1 Aliquot 1mL LCL into each well of PBMC OR

5.5.6.2 Aliquot 5mL LCL per Grex40, then bring volume up to 30Ml

5.5.7 Aliquot 2 to 10mL of LCL alone into an empty well or small flask (as control for efficacy of irradiation).

5.5.8 Culture flasks at 37°C in 5% CO_2 in air for 7 days (proceed to Section 5.5)

5.5.9 Culture GRex40 at 37°C in 5% CO_2 in air for 9 days (proceed to Section 5.5)

5.6 Initiation of antigen-specific CTL using transduced dendritic cells (or transduced LCL)

NOTE: DC are manufactured in Cell Genix medium, but CTLs are initiated in 45% Advanced RPMI, 45% Clicks (EHAA), 10% FCS and 2mM L-glutamine (CTL medium)

5.6.1 Centrifuge PBMC at 400 x G for 5 minutes at ambient temperature.

5.6.2 Remove supernatant and re-suspend at 2 x 10^6 cells per Ml

5.6.3 For ALCI and TGF̃β only add IL-15

5.6.3.1 Add 10ng per mL IL-15 for a final concentration of 5ng per mL (1ul of 10ng per μL stock)

5.6.4 Aliquot 1 mL of cells per well of a 24 well and return plate to incubator

5.6.5 Obtain Ad5f35LMPΔLMP1-I-LMP2 adenovirus vector-transduced DC, irradiated with 30Gy and washed 4 times prepared according to SOP D03.15

5.6.6 If LCL are to be used as antigen-presenting cells, they should have been transduced, irradiated with 40 Gy from a cesium or X-ray source and washed 4 times according to SOP D03.14.

5.6.7 Record removal of LCLs on LCL worksheet

5.6.8 Record LCL production report# on CTL worksheet

5.6.9 Re-suspend antigen presenting cells at 1 x 10^5 (DC) or 5 x 10^4 (LCL) cells/mL.

5.6.10 Aliquot 1ml DC (or LCL) into PBMC wells (a 20:1 ratio of PBMC to DC or 40:1 ratio of PBMC to LCL).

5.6.11 If using LCL, aliquot 2 mL of LCL alone into an empty well (as control for efficacy of irradiation).

5.6.12 Culture at 37°C in 5% CO_2 in air for 7 days (proceed to section 5.5)

5.7 **Initiation of antigen-specific CTLs using adenovirus vector-transduced adherent PBMC**

NOTE: Medium is serum-free X-Vivo15 (monocyte medium)

5.7.1 Day 0. Monocyte activation by adherence to plastic

5.7.1.1 Re-suspend PBMC at 1x10^6 cells per mL

5.7.1.2 Aliquot 2 mL cells per 2 mL well of a 24-well plate or 6 mL per well of a 6 well plate

5.7.1.3 Incubate overnight at 37□C in 5% CO_2 in air.

5.7.2 Day 1 Addition of adenovirus vector

5.7.2.1 Harvest non-adherent PBMCs and use either a cell scraper or a transfer pipette to dislodge adhered monocytes and combine them with non-adherent cells.

5.7.2.2 Count and divide among centrifuge tubes, with up to 1 x 10^7 cells per tube

5.7.2.3 Centrifuge for 5 minutes at 400 X G and aspirate supernatant.

5.7.2.4 Add clinical grade Adenovirus vector to produce the approximate MOI as discussed with the Principal Investigator (see worksheet). For example: If the MOI is 500 viral particles per cell and the adenovirus vector titer is 1 x 10^{12} vp. per mL, then add 5ul of adenovirus vector (diluted 1:10) per 1 x 10^6 PBMCs.

5.7.2.5 Incubate for 120 minutes at 37^{o}C in 5% CO_2 in air.

5.7.2.6 Wash cells 4 times by centrifugation discard supernatant.

5.7.2.7 Re-suspend at 1 x 10^6 cells per mL of **CTL medium**

5.7.2.7.1 45% Advanced RPMI, 45% Clicks (EHAA), 10% FCS and 2mM L-glutamine

5.7.2.8 Aliquot 2ml per well of a 24-well plate.

5.7.2.9 Culture at 37^{o}C in 5% CO_2 in air for 7 days (proceed to section 5.5)

5.7.3 Day 7: Perform a one-half media change for cells in 2 mL wells only

5.7.3.1 Remove ~ 1 mL of medium per well

5.7.3.2 Replace with ~ 1 mL of fresh CTL medium

5.8 2nd Stimulation of EBV or antigen-specific CTLs (Day 9-12)

5.8.1 Harvest responder cells from 24-well plates or GRex40

5.8.2 Count and determine viability according to H05.02

5.8.3 If viability is > 60% proceed to section **5.9 or 5.10**

5.8.4 If viability is less than 60%:

5.8.4.1 Contact PI and centrifuge over Lymphoprep gradients to remove dead cells

5.8.4.2 Harvest interface, centrifuge and re-suspend in medium. Count cells and wash one more time. Proceed if sufficient cells.

5.9 **For 2nd stimulation in G-Rex, transfer to SOP D03.32**

5.10 **For 2nd stimulation in a 24 well plate**

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

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

5.10.3 For EBV-specific CTL

5.10.3.1 Restimulate wiith 40 Gy irradiated, autologous LCL, counted and washed once

5.10.3.2 Note LCL production report# and record removal of LCL worksheet

5.10.4 For antigen-specific CTL

5.10.4.1 Restimulate with adenoviral vector transduced LCL, irradiated 40 Gy, washed 4 times according to SOP D03.14 and counted.

5.10.4.2 Note LCL production report# and record removal of LCL worksheet

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

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

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

5.11 **3-4 day media change with IL-2**

5.11.1 Prepare medium containing IL-2 at 100 to 200 units per mL (final concentration 50 to 100 units per mL) or additional cytokines depending on the Protocol.

5.11.2 Remove ~ 1 mL medium from each well

5.11.3 Culture for 3 to 4 days.

5.12 Disposition of cells after the 2nd stimulation proceed to appropriate SOP(s)

D03.32 Expansion of EBV- or Antigen Specific CTL

D03.33 Retroviral Vector Transduction

D03.21 In process freeze

D03.05 Cryopreservation for infusion

6 Notes

6.1 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.

6.2 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.

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

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

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

7. Expected Results - The cells should have expanded

8. Worksheets

8.1 DW03.31.xA Initiation of EBV-Specific CTL in 24-well plate or GRex40 Bioreactor

8.2 DW03.31.xB Initiation of Antigen-specific CTL using Monocytes

8.3 DW03.31.xC Initiation of Antigen-specific CTL using Adenoviral-Transduced Dendritic Cells (DC)

9. References:

9.1 Leen AM, Sili U, Savoldo B, Jewell AM, Piedra PA, Brenner MK, Rooney CM. Fiber-modified adenoviruses generate subgroup cross-reactive, adenovirus-specific cytotoxic T lymphocytes for therapeutic applications. Blood. 2004 Feb 1;103(3):1011-9.

9.2 Sili U, Huls MH, Davis AR, Gottschalk S, Brenner MK, Heslop HE, Rooney CM. Large-scale expansion of dendritic cell-primed polyclonal human cytotoxic T-lymphocyte lines using lymphoblastoid cell lines for adoptive immunotherapy. J Immunother. 2003 May-Jun;26(3):241-56.

9.3 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

9.4 Bollard CM, Straathof KC, Huls MH, Leen A, Lacuesta K, Davis A, Gottschalk S, Brenner MK, Heslop HE, Rooney CM. The generation and characterization of LMP2-specific CTL for use as adoptive transfer from patients with relapsed EBV-positive Hodgkin disease. J Immunother. 2004 Jul-Aug;27(4):317-27.

REMAINDER OF PAGE LEFT BLANK INTENTIONALLY

10 Review and Revisions:

Written by: *Cliona Rooney*

Reviewed by: *Oumar Diouf*

Malcolm Brenner
Laboratory Medical Director

Adrian Gee
Quality Assurance

Date issued: 07/05/11 Replaces: SOP # D03.31.21

In SOPTrak ☐ Training Forms Issued ☐ Hard copy filed & old version archived ☐

Biannual Review:

2013

	Reviewed without changes	Changed and this version archived
Reviewed by:	_____	_____
QA/QC by:	_____	_____
Date:	_____	_____

In SOPTrak ☐ Training Forms Issued ☐ Hard copy filed & old version archived ☐

2015

	Reviewed without changes	Changed and this version archived
Reviewed by:	_____	_____
QA/QC by:	_____	_____
Date:	_____	_____

In SOPTrak ☐ Training Forms Issued ☐ Hard copy filed & old version archived ☐

2017

	Reviewed without changes	Changed and this version archived
Reviewed by:	_____	_____
QA/QC by:	_____	_____
Date:	_____	_____

In SOPTrak ☐ Training Forms Issued ☐ Hard copy filed & old version archived ☐