Generation of multivirus-specific CTLs

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Method Article

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Abstract

Introduction

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Reagents

RPMI 1640 Invitrogen EHAA \(Click's Medium) Irvine Scientific HI Fetal Calf Serum HyClone L-glutamine \(200 mM) Invitrogen 24-well plate Costar Interleukin-2 \(Proleukin) Chiron \(TCH Pharmacy) Centrifuge tubes Falcon Serological Pipets Falcon Lymphoprep Nycomed Dulbecco's PBS Invitrogen Red Cell Lysis Buffer Becton Dickinson Tissue culture bags American Fluoroseal Connectors B/D X-vivo 15 BioWhittaker

Equipment

Biological safety cabinet, certified Centrifuge Incubator Irradiator Hemacytometer

Procedure

Preparation of mononuclear "responder" cells 1) Dilute heparinized peripheral blood \(ideally 30ml) in an equal volume of D-PBS or RPMI 1640 at ambient \(room) temperature \(or thaw previously frozen PBMCs, wash twice in complete culture media, count, and continue with step 10) 2) In a 50 ml centrifuge tube, carefully overlay approximately 10ml Lymphoprep with 20 ml of diluted blood. 3) Adjust as necessary to utilize all the available cells. 4) Centrifuge at 400 x G for 40 minutes at ambient temperature. 5) Harvest PBMC interface into an equal volume of D-PBS or RPMI 1640. 6) Centrifuge at 450 x G for 10 minutes at room temperature. 7) Remove supernatant. 8) Loosen pellet by "finger-flicking" and resuspend in 20ml of D-PBS or RPMI 1640. 9) Centrifuge at 400 x G for 5 minutes at room temperature. Remove supernatant and resuspend pellet in 20ml complete medium Remove 20ml of cells \(if red blood cell contamination prevents you from obtaining an accurate cell count, add 20ml of 50% red cell lysis buffer) and count. 10) Plate PBMCs at $2x10^6$ cells per well of 24-well plate and incubate overnight at 37°C in 5% CO_2 in X-vivo 15 media. 11) Harvest non-adherent PBMCs and use either a cell scraper or a sterile transfer pipette to dislodge adhered monocytes and transfer to appropriately sized centrifuge tube. 12) Pellet PBMC \(maximum of $6x10^6$/tube) by centrifuging at 400 x G for 5 minutes in a 15ml centrifuge tube, remove supernatant and loosen pellet by "finger-flicking". 13) Add clinical grade Adenovirus pp65 to produce the appropriate multiplicity of infection. For example, if 10 infectious units per cell is required and the Adenovirus pp65 titer is $1x10^{10}$ i.u. per ml, then add 1µl of Adenovirus to $1x10^6$ PBMCs. Incubate for 120 minutes at 37°C/5% CO_2. 14) After 120 minutes, wash cells 4 times by centrifugation at 400 x G for 5 minutes, discard supernatant, loosen pellet by "finger flicking" add complete culture medium so that cells are resuspended at a final concentration of $1x10^6$ per 1ml. 15) Aliquot 2ml per well of 24-well plate. 16) Culture for 9-12 days at 37°C in 5% CO_2. 17) Proceed to second stimulation. **2nd Stimulation:** 1)

Harvest cells from 24-well plates. 2) CTLs should be washed by centrifugation at 400 x G for 5 minutes, discard supernatant, resuspend pellet by ”finger flicking”. 3) CTLs should be resuspended at a concentration of 1 x 10^6 cells/ml of complete medium. 4) If viability is less than 60%, centrifuge over LSM gradients to remove dead cells if necessary 5) Aliquot 1ml cells into wells of a 24 well plate and restimulate with autologous irradiated Ad5f35CMV pp65 transduced LCL \(MOI for transduction is 100 iu/cell) at a responder to stimulator ratio of 4:1 \(2.5x10^5 stimulator cells per well). 6) Culture for 4 days and then feed with a half change of medium. Note: If cells look healthy, wait 4 more days before adding IL-2. 7) At the end of the second stimulation, several aliquots of CTLs \(5 x 10^6) should be cryopreserved as a backup if sufficient cells have been obtained. 8) Centrifuge cells to be frozen @ 400 x G for 5 minutes. 9) Aspirate cell culture supernatant. 10) Resuspend cell pellets by gentle “finger-flicking” 11) Place cells on ice for 10 minutes to cool. 12) Resuspend cells in freezing medium at not greater than 10^7/ml. 13) Distribute aliquots of cells/freezing medium mixture into labeled cryotubes. 14) Place tubes in Nalgene freezing containers and transfer to -80°C freezer and freezer overnight. 15) Transfer to the designated liquid nitrogen freezer for storage. **After the second stimulation, cells are expanded as follows:** 1) Cells are stimulated twice a week with 40-100 units/ml of IL-2 according to expansion rate. Concentrations at the upper end of the range should be used for cells that have not doubled in number. 2) Weekly, cells are harvested, pooled, counted and replated in at 1 x 10^6 responder cells per well in a 24 well plate or at 1 x 10^6 responder cells per mL in a bag. 3) They are then restimulated with irradiated transduced autologous LCL at a responder to stimulator ratio of 4:1. 4) If the CTL line is growing rapidly, it may require splitting on the day of the IL-2 feed. If there are more than 3 x 10^6 cells per well, the cells should be re-plated at 2 x 10^6 cells per well. 5) When sufficient cells for patient infusion \(according to dose and Body Surface Area), the cells should be characterized and frozen. 6) Within one week of freezing CTLs, cytotoxicity assays should be set up with 5 x 10^6 cells and phenotyping should be done with an additional 2 x 10^6 cells.

Anticipated Results

The cells should have the following characteristics: Sufficient CTL numbers for infusion of the patient at the appropriate dose level \(determined at that time) and for all QC requirements. < 10% killing of recipient PHA blasts or fibroblasts <2% CD19+ B cells by flow cytometry \(exclusion of LCL) <2% CD14+ cells \(exclusion of monocytes)

References

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