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Ex vivo generation and maintenance of human Epstein-Barr Virus (EBV)-specific T cells lacking alloreactivity

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ABSTRACT

T cells recognizing Epstein-Barr Virus (EBV) can be generated ex vivo from most immunocompetent human subjects and maintained in vitro for extended periods of time. EBV T cells generally display viral antigen-specificity, high cytolytic and inflammatory capacity, and when repeatedly sensitized to autologous EBV blasts, lack xeno- or alloreactivity. Thus, EBV T cells are of high utility for in vitro and in vivo studies involving human T cell transduction, e.g. of a CAR or TCR, or as responder cells for assaying the activity of T cell-specific immunomodulatory agents such as bispecific antibodies or BiTEs. EBV T cells have also been used clinically to treat EBV+ malignancies in several independently conducted studies. This protocol details the basic in vitro steps necessary to generate EBV T cells to supplement what is described in the expansive literature making use of these cells.

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Materials

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- **Human PBMC**
- **EBV B95-8 transformed B cells (BLCL**) from the same donor as the PBMC (protocol here)
- **Human IL-2**
- **T cell media**: specialty medium (Yssel's, Xvivo-15 or -20, AIM-V, OpTimizer, CellGro DC media) supplemented with 5-10% human AB serum and 2mM L-Glutamine *OR* basal medium (RPMI 1640, IMDM) supplemented with 5-10% human serum, 2mM L-Glutamine, non-essential amino acids, and 2-mercaptoethanol. Even though culture medium is rich in amino acids, L-Glutamine should still be added from frozen working stocks to each batch of T cell media as it is not stable for long in solution at 4C. Specialty medium has the added advantage of being able to keep cells happy even at high density.
- **Gamma irradiator**

Procedure

3 **Day 0 - Primary stimulation:**

1. Thaw cryopreserved or freshly isolated PBMC from the same donor as the BLCLs. Determine the number of PBMCs by counting viable nucleated cells with a defined border. Cells lacking defined round edges should not counted as they will die off.
2. Determine the number of BLCLs you will use as feeders based off of a 20:1 PBMC:BLCL ratio (also called Responder:Stimulator or Effector:Target ratio)
3. Irradiate the required number of BLCLs at 90 Gy
4. Adjust the PBMCs to 1-2e6 cells/mL in T cell media
5. Spin down the irradiated BLCLs and resuspend them in the PBMC suspension
6. Incubate the cells in an appropriate sized vessel based on the volume of the suspension: 6-12mL in a T12 flask, 12.5-20mL in a T25 flask, 25-35mL in a T75 flask, 35-60 mL in a T175, 60-100mL in a T225.
7. Incubate cells 7-10 days at 37C

Day 7-10 - Secondary stimulation:

1. On day 7-10, count the cells. The % of viable cells will vary depending on how much the responding lymphocytes outgrew the rest of the dying PBMCs. If the density of viable cells is >1-2e6 cells/mL (higher than the initial seeding density) and media is acidified, subculture the cells at a density of 0.8-1e6 cells/mL by resuspending entire culture in fresh media at the appropriate volume. If the density is <1-2e6 cells/mL and media has not acidified, harvest half of the culture, spin it down, and resuspend it with the remaining culture as its media has been valuably conditioned by the lymphocytes. Add fresh media as needed to adjust the concentration to 1e6 cells/mL.
2. Add irradiated BLCL at a 4:1 responder:stimulator ratio based off the number of viable cells in the culture.
3. Incubate 3 days

Day 10-13 onwards:

1. Three days after restimulation, add 20 U/mL IL2.
2. 2-3 days later, add 20U/mL IL2 to the culture. Add IL2 at this dose 3x a week. Always assume all prior IL2 was consumed and base the dose off entire culture volume.

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3. Restimulate cells 7-10 days after the last restimulation with BLCL at the same 4:1 R:S ratio, adding IL2 3 days later. Maintain cells at a working concentration of 0.8-1e6 cells/mL.
4. If cell numbers drop after secondary stimulation or later restimulation with BLCL, increase the IL2 dose by 20-50 U/mL increments. Ex. cells are restimulated on day 10 as described above, and on day 13 the cell density is has not changed or decreased. Increase the IL2 dose to 40 U/mL on this day, and every subsequent IL2 feeding. If this happens 3 days after the next stimulation, the IL2 dose will again be increased to 60-100 U/mL and all subsequent feedings will be at this dose.
5. Maintain autologous BLCL restimulations every 7-10 days, keep cells at 0.8-1e6 cells/mL, and feed IL2 three times a week to maintain cells.

Quality control

4 There are a few QC checks that should be regularly performed for your culture of EBV T cells, ideally once every stimulation starting after the 3rd or 4th stimulation. Your EBV T cells failing any of these QC checks can mostly be attributed to variability between donors as each culture is different due to donor-dependent differences in BLCL HLA expression and EBV T cell subset precursor frequency.

- **Immunophenotype**: check CD3, CD4, CD8, CD56 by flow cytometry. CD56+CD3- cells can grow in your culture due to NK cell consumption of gamma chain cytokines like IL2. If you find CD56+ cells outcompeting T cells in your culture, you can deplete them using CD56 beads or by FACS. The CD4+CD3+:CD8+CD3+ ratio will be dependent on the donor used.
- **Cytotoxicity**: your EBV T cells should display HLA-dependent cytolytic activity against the autologous BLCLs. This can be determined by a number of assays including 51Cr, LDH, intracellular caspase-3 staining, or 7AAD/Annexin V staining. Autologous BLCLs in the presence of HLA-blocking antibodies like W6/32 and IVA12 should not be killed.
- **Antigen specificity**: your EBV T cells should have specificity for antigenic peptides from EBV such as the EBNAs or LMPs. The HLA-restriction of this specificity and proportion of cells responding to particular antigenic peptides is completely donor-dependent, even between donors sharing HLA alleles. Antigen specificity can be assayed for in killing assays as well as cytokine-based assays (ELISPOT, intracellular cytokine staining, ELISA, Luminex) using peptide-pulsed targets bearing relevant HLA alleles. For determining HLA restriction, it is useful to assemble a panel of target BLCLs expressing relevant and irrelevant HLA alleles to be used in killing assays, such as the IHW cell lines.

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