Abstract

Viral infections cause morbidity and mortality in allogeneic hematopoietic stem cell transplant (HSCT) recipients. We and others have successfully generated and infused T-cells specific for Epstein Barr virus (EBV), cytomegalovirus (CMV) and Adenovirus (Adv) using monocytes and EBV-transformed lymphoblastoid cell (EBV-LCL) gene-modified with an adenovirus vector as antigen presenting cells (APCs). As few as 2x10^5/kg trivirus-specific cytotoxic T lymphocytes (CTL) proliferated by several logs after infusion and appeared to prevent and treat even severe viral disease resistant to other available therapies. The broader implementation of this encouraging approach is limited by high production costs, complexity of manufacture and the prolonged time (4-6 weeks for EBV-LCL generation, and 4-8 weeks for CTL manufacture – total 10-14 weeks) for preparation. To overcome these limitations we have developed a new, GMP-compliant CTL production protocol. First, in place of adenovectors to stimulate T-cells we use dendritic cells (DCs) nucleofected with DNA plasmids encoding LMP2, EBNA1 and BZLF1 (EBV), Hexon and Penton (Adv), and pp65 and IE1 (CMV) as antigen-presenting cells. These APCs receive T cells specific for all the stimulating antigens. Second, culture of activated T-cells in the presence of IL-4 (1,000U/ml) and IL-7 (10ng/ml) increases and sustains the repertoire and frequency of specific T cells in our lines. Third, we have used a new, gas permeable culture device (G-Rex) that promotes the expansion and survival of large cell numbers after a single stimulation, thus removing the requirement for EBV-LCLs and reducing technician intervention. By implementing these changes we can now produce multispecific CTL targeting EBV, CMV, and Adv at a cost per 10^6 cells that is reduced by >90%, and in just 10 days rather than 10 weeks using an approach that may be extended to additional protective viral antigens. Our FDA-approved approach should be of value for prophylactic and treatment applications for high risk allogeneic HSCT recipients.

Protocol

1. **DC nucleofection**

   1. Harvest monocyte-derived DCs, which have been enriched using plastic adherence, cultured for 5 days using Cell Genix Media supplemented with IL4(1000U/ml), GMCSF (800IU/ml) and further matured for 24hrs using the DC maturation cytokines IL4(1000U/ml), GMCSF (800IU/ml), IL10(ng/ml), TNF-α 10ng/ml, IL1-β 10ng/ml and PGE2 (1ug/ml), by gentle resuspension with a 3ml transfer pipette.
   2. Count viable DCs using trypan blue, transfer into 3x 15ml tubes with no fewer than 0.5x10^6 and no more than 2x10^6 cells/tube.
   3. Centrifuge DCs for 10mins @ 200g. During this time pre-warm Cell Genix media supplemented with the DC maturation cytokines (DC maturation media) - 2ml/well in three wells of a 12-well tissue culture treated plate in a 37°C/5% CO2 incubator.
   4. Once cells have finished spinning, aspirate the supernatant and add the relevant DNA plasmids to each of the tubes in a final concentration of 5µg DNA/tube. In this case add the plasmid encoding IE1-pp65 to tube #1, Hexon-Penton to tube #2, and EBNA1-LMP2-BZLF1 to tube #3.
   5. Resuspend DCs and DNA with 100µl of Amaxa nucleofection solution, mix well and transfer to the nucleofection cuvettes.
   6. Place cuvettes in the 4D nucleofector, choose program CB150 (Amaxa/Lonza), and press start.
   7. Immediately after nucleofection add 500µl of the 2ml pre-warmed Cell Genix DC maturation media to the cuvette, mix gently by pipetting up and down 2-3 times, and transfer nucleofected DCs to the prepared 12-well plate containing the remaining 1.5 ml of prewarmed DC maturation media. Transfer to the 37°C/5% CO2 incubator for a further 12-18hrs.

2. **T cell stimulation**

   1. Harvest and count nucleofected DCs, and irradiate at 30Gy. Wash once with 10ml of CTL medium (45% RPMI, 45% Clicks EHAA, 10% FBS, 2mM Glutamax) and resuspend @ 3 x 10^6 DCs per ml of CTL media.
   2. Pool a minimum of 5.5x10^9 (2.5ml) and a maximum of 15x10^9 (5ml) of DCs containing each of the plasmids and transfer the pooled DCs to the G-Rex device which will then be placed in the incubator.
   3. For the preparation of responder cells use either previously frozen PBMCs or non-adherent mononuclear cells that remain after the DC selection (adherence or CD14 selection). Thaw the cells, transfer to prewarmed culture medium, wash once with CTL media. Resuspend the cells in CTL Media, count the cells and bring them to a concentration of 2x10^6 cells per ml. Take 15x10^6 cells or 7.5ml of and supplement with 30000U IL4 (1000U/ml – final conc.) and 300ng IL7 (10ng/ml – final conc.).
   4. Transfer 7.5ml of PBMC (15x10^6 cells) to the G-Rex and top up the bioreactor with CTL media to a total volume of 30ml.
   5. Culture the G-Rex for 6-7 days in a 37°C/5% CO2 humidified incubator.

3. **T cell expansion**

   1. On day 6-7, aspirate 10ml of media, then mix the cells in the remaining 20ml of media with a 10ml pipette and count viable cells using trypan blue. If there are <5x10^6 replenish with fresh media + cytokines. If there are >50x10^6 cells remove 10ml of cell suspension, transfer to a new G-Rex, and then feed both G-Rexs with fresh CTL media + cytokines.
4. Representative Results:

A schematic of our FDA-approved multivirus-specific CTL generation process is shown in Figure 1. In contrast to convention multivirus CTL protocols which use adenovectors and EBV-LCL to stimulate virus-reactive T cells, we have replaced infectious virus material with DNA plasmids that encode multiple antigens derived from each of the viruses. To stimulate trivirus CTL, we designed three multicistronic plasmids encoding Hexon and Penton of adenovirus, IE1 and pp65 of CMV, and EBNA1, LMP2, and BZLF1 of EBV. These antigens were chosen based on encouraging clinical results of our own and other groups showing that T cells directed against Adv-hexon and penton, and to CMV-IE1 and to CMV-pp65 are protective in vivo. For EBV, EBNA1 is an immunodominant CD4+ T cell target antigen expressed in all EBV-associated malignancies and in normal EBV-infected B cells. LMP2 is immunogenic across multiple HLA types and expressed in most EBV malignancies, while BZLF1 encodes an immunodominant, immediate early lytic cycle antigen that stimulates both CD4+ and CD8+ T cells from most individuals and is likely important for the control of cells replicating virus. To further optimize our manufacturing methods, we collaborated with Nature Technology who generated minimalized, antibiotic-free (FDA-compliant) plasmids for CTL stimulation. Using this strategy, we consistently achieve nucleofection efficiencies of >35% while maintaining high cell viability (data not shown). Figure 2 shows that the frequency of virus-specific T cells in response to optimized DNA plasmids as measured by IFNγ ELIspot, was greater than in response to conventional pShuttle-based expression plasmids expressing the same antigens. The optimal ratio of DC:PBMC was important for potent T cell stimulation as shown in Figure 3 where a ratio of 1:50 produced sub-optimal activation compared to a 1:20 S:R ratio. Production of sufficient CTL numbers with broad antigen specificity is a prerequisite for clinical efficacy against all three viruses. This is achieved by CTL culture in the G-Rex, which supports superior T cell expansion compared with conventional 24-well plates, while the addition of IL4 and IL7 to cultures increases the repertoire and specificity as shown in Figure 4B where the frequency of T cells reactive against the CMV-pp65-derived HLA-A2 restricted NLV peptide was assessed in cultures generated in the presence or absence of IL4 and/or IL7. To assess the phenotype and functional capacity of the expanded cells, we perform flow cytometric analysis, intracellular cytokine staining/IFNγ ELIspot, and Cr51 release assays on the final product for cryopreservation/infusion. Typically the generated cells are polyclonal with a mixed population of CD4+ and CD8+ T cells with antigen-specificity detectable in both T cell compartments. The CTL are able to kill viral antigen-expressing target cells but not virus negative partially-HLA matched targets, indicating that they should not induce graft-versus-host disease (GvHD) in vivo (Figure 5).
Figure 2. Optimized DNA plasmids induce superior T cell activation in vitro. DCs were nucleofected with optimized, FDA-compliant plasmids encoding Hexon and Penton (Adv), IE1 and pp65 (CMV), and EBNA1, LMP2, and BZLF1 (EBV) or conventional pShuttle plasmids encoding the same antigens. These were used to stimulate T cells and specificity was analyzed by IFNγ ELISPOT 10 days post-stimulation.

Figure 3. Optimal DC:T cell ratios for CTL activation. DCs from 2 donors were nucleofected with all three optimized plasmids and then used to stimulate autologous PBMCs at 1:20 or 1:50 DC:PBMC ratio. The frequency of reactivated T cells was assessed on day 10 by IFNγ ELISPOT.
Figure 4. T cell expansion in the G-Rex using enhancing cytokines. Panel A shows the G-Rex device as well as CTL appearance on the gas permeable membrane, evaluated by microscopy. A comparison between cell output achieved in convention tissue culture treated plates vs G-Rex is also shown. Panel B shows the frequency of CMV pentamer positive CTL achieved in cultures expanded in the presence of no cytokine, IL4 alone, IL7 alone and IL4+IL7.

Figure 5. Phenotype and function of expanded CTL. Panel A shows a representative example of the phenotype of the expanded multivirus CTL, which are polyclonal with a mixture of CD4+ (45% - helper) and CD8+ (42% - cytotoxic) T cells, of which the majority (95%) expressed the memory marker CD45RO+/CD62L+. Panel B shows that these cells are specific for all the stimulating antigens and are polyfunctional as assessed by intracellular cytokine staining to detect production of IFNγ and TNFα after antigen stimulation. Panel C shows that the expanded
CTL are functional as measured by Cr⁴⁺ assay. Autologous LCL, either alone or transduced with a null vector or an adenoviral vector expressing CMV-p65 were used as targets. Alloreactivity was assessed using allogeneic PHA blasts as a target.

Discussion

Viral infections account for substantial morbidity and mortality in patients who are immunocompromised by their disease or its treatment. After HSCT, for example, infections caused by persistent herpesviruses such as EBV and CMV, as well as by respiratory viruses such as Respiratory Syncytial Virus (RSV), are well known, while the importance of infections caused by Adv, BK virus, and human herpesvirus (HHV)-6 have more recently been appreciated. While pharmacological agents are standard therapy for some infections, they have substantial toxicities, generate resistant variants, and are frequently ineffective. In contrast, virus-specific T cells derived from stem cell donors have proven safe and effective for the prevention and treatment of viral infection or disease in the hematopoietic stem cell transplant (HSCT) setting²,5,10-18. However, the broader implementation of T cell immunotherapy is ultimately limited by the cost, complexity and time required for CTL production.

Our novel and rapid approach to generate multivirus CTL, described in the current manuscript, should substantially increase the feasibility of cytotoxic T cell therapy for viral diseases, enabling the strategy to become a standard of care for the immunocompromised host. The use of plasmid nucleofected DCs as APCs enables antigen presentation on both MHC class I and II without competition from viral vectors or indeed from multiple viral antigens being expressed within a single cell since different DC populations are utilized for each plasmid⁸. The use of IL-4/7 increases T cell survival and proliferation, which correspondingly helps increase the frequency and repertoire of responding antigen-specific T cells¹⁶,¹⁷. Finally, culture in the G-Rex dramatically reduces T cell apoptosis during culture. Gas exchange (O₂ in and CO₂ out) occurs across a gas permeable silicon membrane at the base of the flask, preventing hypoxia while allowing a greater depth of medium above the cells, providing more nutrients and diluting waste products. This platform can also be extended to additional viruses as when protective antigens are identified.

Disclosures

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