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Review Article

Optimization Manufacture of Virus- and Tumor-Specific T Cells

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Although *ex vivo* expanded T cells are currently widely used in pre-clinical and clinical trials, the complexity of manufacture remains a major impediment for broader application. In this review we discuss current protocols for the *ex vivo* expansion of virus- and tumor-specific T cells and describe our experience in manufacture optimization using a gas-permeable static culture flask (G-Rex). This innovative device has revolutionized the manufacture process by allowing us to increase cell yields while decreasing the frequency of cell manipulation and *in vitro* culture time. It is now being used in good manufacturing practice (GMP) facilities for clinical cell production in our institution as well as many others in the US and worldwide.

1. Introduction—T Cell Transfer

Cell therapy is a new but rapidly expanding field in biotechnology which involves the administration of autologous or allogeneic cells that carry out a therapeutic effect *in vivo*. The first adoptive T cell transfer protocols in the allogeneic hematopoietic stem cell transplant (HSCT) setting were based on the premise that donor peripheral blood contained T cells able to mediate antitumor and/or antiviral activity in the HSCT recipient. Accordingly, donor lymphocyte infusions (DLIs) have been extensively used to provide both antitumor and antiviral immunity. However, the relatively high frequency of alloreactive cells compared with virus- and/or tumor-specific T cells results in a significant incidence of graft-versus-host disease (GvHD), thereby limiting the applicability of this approach. Infusion of enriched antigen-specific T cells with reactivity against a particular antigen potentially increases therapeutic potency while decreasing undesired "off-target" effects or GvHD, and this field has grown over the past two decades. This paper focuses on the production of *in vitro* expanded antigen-specific T cells, discusses conventional and current technologies for T cell generation, and outlines recent advances in cell production techniques which may ultimately move this therapeutic modality from a boutique application towards a "standard of care."

2. Infusion of *Ex Vivo* Expanded CTL

The infusion of *in vitro* expanded donor-derived virus-directed cytotoxic T lymphocytes (CTLs) targeting one (Epstein-Barr virus (EBV)), two (EBV and Adenovirus (Adv)), or three viruses (EBV, Adv, cytomegalovirus (CMV)) has proven to be safe, effective, and protective *in vivo* [1–4]. The adoptive transfer of tumor antigen-directed T cells has also induced objective tumor responses and complete remissions in patients with advanced lymphoma, melanoma, and nasopharyngeal carcinoma [5–10]. Recent advances in molecular biology techniques have increased the enthusiasm for this therapeutic modality by (1) allowing the genetic modification of T cells with a wide range of genes which confer new antigen specificity by transferring T cell receptors (TCRs) or chimeric antigen receptors (CARs) [11–14], (2) improving the homing and proliferative properties of effector cells [15, 16], and (3) controlling unwanted T cell proliferation or *in vivo* activity [12, 17–20].

Although the administration of *in vitro* expanded antigen-specific CTLs has produced promising clinical results, there are several factors limiting the extension of this approach beyond the research arena. A major practical constraint is the current complexity associated with production of large number of cells using traditional manufacture

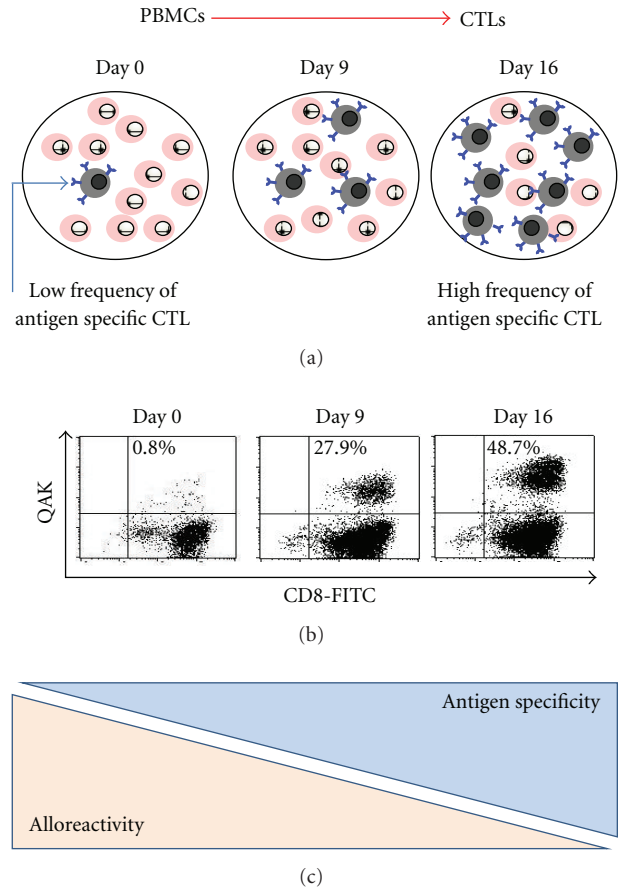


Figure 1: Increased frequency of antigen-specific CTLs after *in vitro* stimulation. (a) illustrates the low frequency of antigen-specific CTLs present in peripheral blood and the subsequent enrichment after antigen stimulation. (b) shows the enrichment of QAKWRLQTL- (HLA-B8-restricted EBV epitope-) specific T cells in a seropositive donor as evaluated by tetramer analysis. (c) illustrates the inverse correlation between the frequency of antigen-specific and alloreactive T cells in peripheral blood (left) and in vitro expanded CTLs (right).

protocols. However, some recent advancements streamlined the production process.

3. *Ex vivo* Expansion of Antigen-Specific T Cells

The *ex vivo* generation of antigen-specific T cells is conventionally accomplished by repeat in vitro stimulation with professional or artificial antigen presenting cells (APCs) which express the protein or peptide of interest and culture in the presence of cytokines which promote T cell proliferation, such as interleukin- (IL-) 2 [1, 21, 22]. This process results in the amplification and enrichment of T cells directed against the stimulating antigen/peptide with a corresponding decrease in the frequency of cells with undesired specificities such as alloreactive T cells (Figure 1). Once sufficient cells (required for adoptive transfer) are generated, these are then tested for potency, purity, identity, and sterility prior to infusion.

For example, EBV-specific CTLs can be expanded *ex vivo* from EBV-specific T cell precursors generally present at a frequency of up to 1% in the peripheral blood of most seropositive individuals. Traditionally, enriched T cell lines are prepared by coculturing 1×10^6 peripheral blood mononuclear cells (PBMCs) per cm^2 with gamma-irradiated (40 Gy) autologous EBV-transformed lymphoblastoid cell lines (EBV-LCLs) at a 40 : 1 ratio (PBMC : LCLs) in a total volume/well (of a tissue culture treated 24-well plate) of 2 mL CTL growth media (RPMI 1640 supplemented with 45% Click medium (Irvine Scientific, Santa Ana, Calif), 2 mM GlutaMAX-I, and 10% FBS). Between days 9 and 12 CTLs are harvested, counted, resuspended in fresh media, re-seeded at 5×10^6 per cm^2 in a total volume of 2 mL of CTL media, and then fed with recombinant IL-2 (50 U/mL) 4 days later. This initial 13–16-day culture period in the absence of exogenous cytokines gives a proliferative/survival advantage to the small population of EBV-specific T cells present in PBMCs, which both produce and use IL-2 in an autocrine manner upon stimulation with EBV-LCL. However, at later time points, when cultures are exclusively EBV specific the level of available cytokine becomes limiting and thus cultures must be supplemented to ensure that CTL proliferation is adequately supported [23]. Subsequent stimulations are performed every 7 days using a 4 : 1 CTL:EBV-LCL ratio with twice weekly addition of IL-2 (50 U/mL). This *ex vivo* propagation of EBV-specific T cells continues until sufficient cells are generated for cryopreservation and quality control analysis including HLA typing to confirm identity, purity, and safety testing. All products must meet the specified release criteria before they are released for infusion. Additional analysis on specific products such as assessment of transgene expression may also be performed. For example, one of the release criteria for chimeric-antigen-receptor- (CAR-) modified EBV-CTLs is that at least 15% of cells must express the transgene. Though there are different CTL generation protocols used by different groups, even for the generation of the "same" product, the component parts/core requirements (antigen, APC, and cytokine) are essentially the same.

4. Traditional *in vitro* Culture of Antigen-Specific T cells

A large variety of manufacturing protocols have been described for the *in vitro* expansion of T cells. Small numbers of suspension cells ($<5 \times 10^7$) can be relatively easily propagated using conventional multiwell tissue culture treated plates or flasks. However, when the number of cells required exceeds the maximum capacity of a single plate or flask (e.g., $>5 \times 10^7$) this platform becomes time consuming and cumbersome to manipulate.

Cell propagation *in vitro* is limited by requirements for nutrients and oxygen (O_2) and by the accumulation of metabolic waste such as lactic acid and carbon dioxide (CO_2). Cell culture in conventional cultureware is restricted to the use of specific media volumes per surface area unit, that is, a maximum of 1 mL media should be added per cm^2 since this is permissive to gas diffusion. However, this shallow

media volume limits both the available nutrients and the buffering capacity of the media. In addition, as cell numbers increase, O_2 and nutrient requirements progressively increase, so that cultures must be fed and re-seeded regularly. These frequent medium changes and cell manipulations are time consuming and expensive, reduce the reproducibility of cell production, and increase the risk of contamination.

5. Alternative Vessels for T Cell Expansion

One way to overcome the limitations associated with scale-up using conventional cultureware is to instead utilize a cell bioreactor that provides mechanical rocking or stirring to perfuse media with gas. The use of such bioreactors augments cell expansion, resulting in higher cell densities beyond that attained using conventional plasticware.

A large number of bioreactors (hollow fiber bioreactors, stirred tank bioreactors, and WAVE bioreactors) have been explored for the expansion of suspension cells such as activated T cells, genetically modified T cells, or antigen-specific CTL [23–27]. In these bioreactors oxygen is provided by mechanical rocking or stirring or by pumping gas through the culture while medium can be exchanged by perfusion. Stirred bioreactors allow excellent gas exchange and can be scaled up relatively easy. However, shear stress associated with the stirring rate adversely affects cell viability and thus it has not been broadly adapted [28]. In contrast, hollow fiber bioreactors allow a constant perfusion of the culture, thus diluting metabolites without shear stress. However, accessibility to this device makes it difficult to efficiently recover the expanded cells [24]. Static culture bags limit the achieved cell densities (per input media volume). Thus, the generation of large cell numbers requires the use of large media volumes with a resultant increase in the frequency of manipulations required to obtain the final product [29]. Although the WAVE Bioreactor has been effectively adapted for the expansion of primary T cells, resulting in the generation of large numbers of cells (10^{15}), the culture bag cannot be accommodated in a standard incubator and must be heated and rocked in an expensive, custom-made device [30, 31]. In addition, optimal cell growth is maintained by regular measurement of oxygen and lactic acid and a peristaltic pump is needed to move medium in and out of the bag, necessitating the incorporation of special filters to prevent cells being damaged by the pump. Further, gas is propelled through the culture using a control flow meter which ensures that culture osmolarity is maintained.

Although antigen nonspecific T cell cultures have been grown with some success in these various bioreactors, antigen-specific T cells have strict requirements for cell-to-cell contact and have proven difficult to consistently adapt to moving cultures. Therefore many groups, including our own, have found it difficult to improve upon results achieved using the 2 cm^2 wells of standard tissue culture-treated 24-well plates, which are ideal for the expansion of small numbers of cells required for preclinical and proof of concept studies but limit the translation of antigen-specific T-cell-based therapies beyond the academic level (Figure 2). Table 1 shows the relative advantages and disadvantages associated with each of the culture vessels which have been used to produce T cell products for clinical applications.

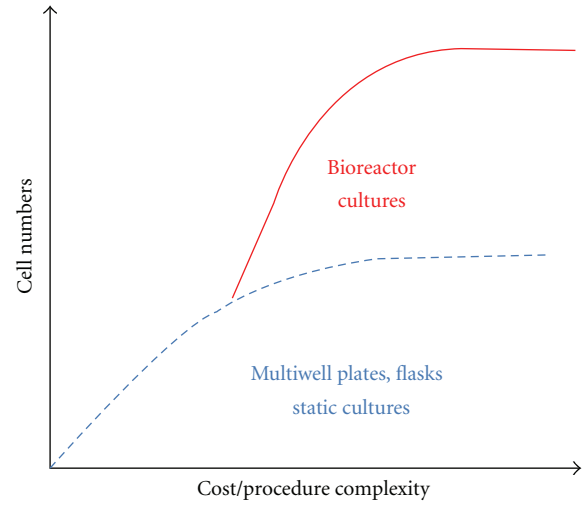


Figure 2: Increased cost and procedure complexity with large-scale cell requirements. As illustrated multiwell plates or flasks are ideal for the expansion of small numbers of antigen-specific CTLs ($<5 \times 10^7$). However, this system becomes ineffective for the expansion of large numbers of cells. In contrast cell bioreactors are ideal for the production of large cell numbers, but this platform is difficult to adapt and requires specialized equipment.

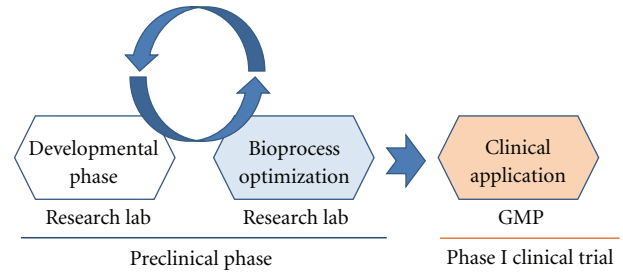


Figure 3: Dynamic bioprocess optimization. This dynamic interaction between the optimization and the preclinical phase allows for easy transition of a cell product into the cGMP.

6. Dynamic Bioprocess Optimization

The problem with most manufacturing processes is the misconception that a product can be produced on a large scale by simply using a linear scale-up model. In most cases this is simply not feasible given that the production protocols are, for the most part, specialized, highly complicated, and convoluted. One way to overcome this scale-up problem, which is a bottleneck in conventional cellular therapies, is to incorporate bioprocess optimization in the manufacturing process. That will ultimately pave the way for an easy transition into the GMP and will almost guarantee manufacturing success, thus positively impacting the outcome of a clinical study. This bioprocess optimization (as illustrated in Figure 3) should not be considered

Table 1: Suitability and properties of different culture vessels for T cell expansion.

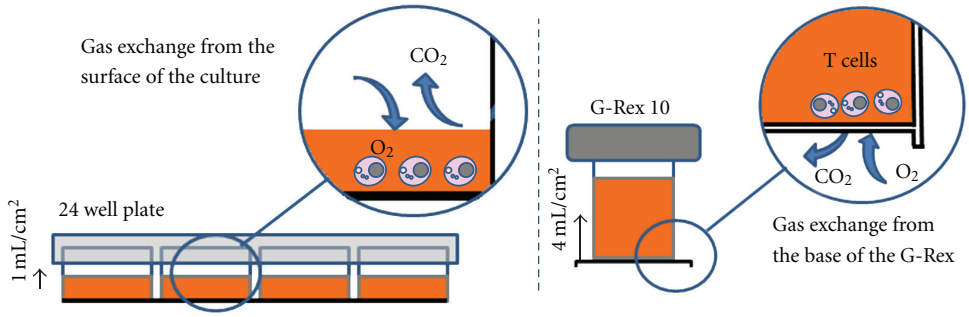
Cell culture vessels	Gas exchange	Volume of media	Cell concentration	Disadvantages	Advantages
Multiwell plates/flasks (static cultures)	Limited	Limited: low ratio of medium to surface area	Low	High risk of contamination Extensive processing time Frequent interventions Not scalable	Suitable for small-scale cell production
Gas-permeable bags (static cultures)	Good	Limited: low ratio of medium to surface area	Medium	Low output per bag requires constant culture maintenance Limited microscopic cell examination Not linearly scalable from research to production	Sterility of closed system
G-Rex (gas-permeable static cultures)	Excellent	Unrestricted: high ratio of medium to surface area	High	Limited microscopic cell examination	Excellent O_2 exchange Linearly scalable from research to large-scale production Significantly reduced culture manipulation Compatible with closed system
Wave action bioreactors with CO_2/O_2 aeration & pH controllers (dynamic cultures)	Good	Unrestricted: high medium capacity in each bag	High	Complex, costly, requires special equipment. Not well suited to coculture stage of CTL production Requires constant culture maintenance. Limited microscopic cell examination Not linearly scalable from research to large-scale production	Excellent O_2 exchange yields large cell numbers Closed system

a "validation stage" but instead a dynamic interaction between the preclinical phase and manufacturing optimization that seeks to simplify the product generation, while ensuring that the cell product maintains the biological properties achieved in small scale manufacture.

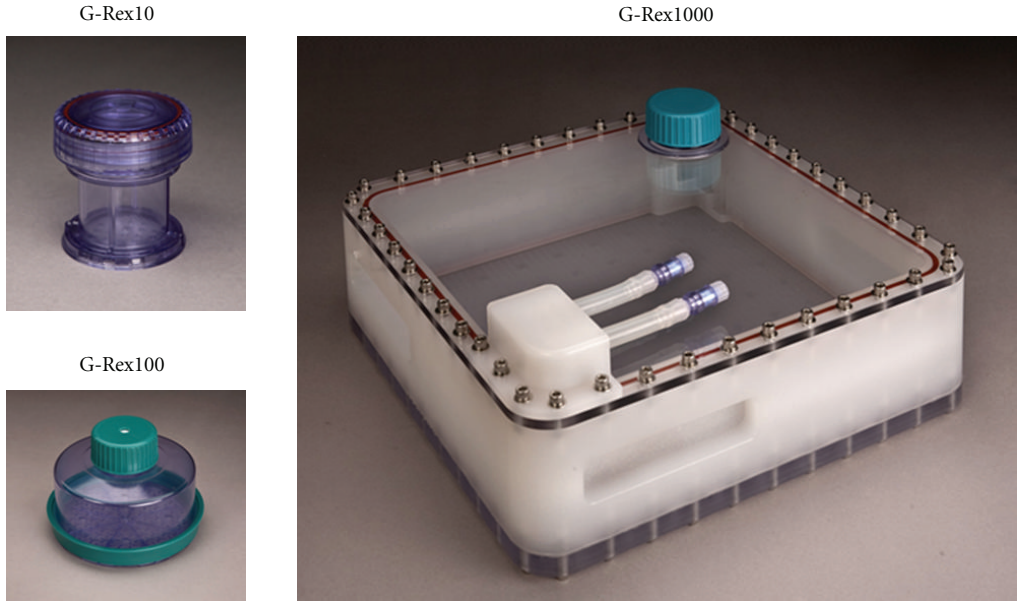
7. Our Experience

One example of manufacture optimization that we have undertaken over the past 4 years at the Center for Cell and Gene Therapy (CAGT) at Baylor College of Medicine and supported by Production Assistance for *Cellular Therapies* (PACT) surrounds our search for simpler and more rapid strategies to expand antigen-specific T cells for adoptive transfer. Traditionally our group and others have cultured virus- and tumor-directed T cells in 2 cm^2 wells of tissue culture treated 24-well plates. These T cells are often propagated for 8 weeks or longer to achieve the cell numbers required for clinical application. However, the restricted media ratio (1 mL/cm^2) associated with gas diffusion limits the supply of nutrients, which are rapidly consumed by proliferating T cells. Consequent acidic pH and waste build-up rapidly impedes cell growth and survival. Therefore, the only alternative for cell propagation is frequent reseeding and medium exchange which increases the frequency of manipulation required with a concomitant increase in the risk of contamination. Thus, we sought to optimize our antigen-specific T cell culture process which led us to evaluate a novel cell culture device (gas-permeable cultureware (G-Rex)), developed by *Wilson Wolf Manufacturing*, and in which O_2 and CO_2 are exchanged across a silicone membrane at the base of the flask. Because gas exchange occurs from below this allows an increased depth of medium above, which provides more nutrients required by the cells while waste products are diluted, thus not adversely affecting cell growth (Figure 4).

These optimal culture conditions provided by the G-Rex result in improved cell viability and increased final cell numbers without increasing the number of cell doublings, and decreasing the feeding frequency and the number of manipulations required [32]. For example, for the expansion of EBV-CTLs using the G-Rex we co-culture 1×10^6 PBMCs per cm^2 using a G-Rex10 (surface area of 10 cm^2—total



(a)



(b)

Figure 4: G-Rex culture device. (a) shows the limited gas exchange that occurs in conventional cultureware, which limits the volume of media and consequently the available nutrients. In contrast the G-Rex provides gas exchange from the base of the flask which allows cells to be cultured with a superior ratio of media per surface area. (b) shows the G-Rex10 with a surface area of 10 cm^2 and a volume capacity of 40 mLs, the G-Rex100 with a surface area of 100 cm^2 and a volume capacity of 500 mLs, and the G-Rex1000 with a surface area of 1000 cm^2 and a volume capacity of 5000 mLs.

of 1×10^7 PBMCs) with gamma-irradiated (40 Gy) EBV-LCLs at a 40 : 1 ratio in a final volume of 40 mL of CTL medium. On days 9–12 the second stimulation is performed by removing 20 mL of media (aspirated from the top) and adding 20 mLs of fresh CTL medium containing irradiated EBV-LCLs, resuspended at a cell density appropriate to stimulate T cells at a ratio 4 : 1. Four days after the second stimulation 50 U/mL of IL-2 is added directly to the culture. Once the cells have expanded to a density of $>5 \times 10^6$ per cm^2 the cells are transferred to a G-Rex100 (surface area 100 cm^2) and stimulated with irradiated EBV-LCL (4 : 1) in a final volume of 500 mLs of media. These culture conditions have allowed us to decrease the frequency of culture manipulation while increasing the cell output (3–20-fold) and shortening the time of culture [32] (Figure 5). We demonstrated that this novel culture system supports the expansion of almost any type of suspension cell, is GMP-compliant, and reduces the number of technician interventions approximately 4-fold [32].

This manufacture optimization has been validated, transferred to our GMP facility in 2009 and is now used for all of our CTL production processes. Since that time we have allowed other centers, including the NCI, to cross-reference our IND to enable the use of this cell culture technology in other GMP facilities both within the US and beyond, and this platform is currently used for production of numerous cellular products including activated T cells, antigen-specific CTLs, NK cells, regulatory T cells, and feeder cells including EBV-LCLs and aK562 [32]. Importantly, cell culture in the G-Rex can also be linearly scaled which allows an easy transition of protocols from small to large scale. We recently demonstrated this using the new G-Rex600 and G-Rex1000 (surface area of 600 and 1000 cm^2, resp.), which can generate up to 6×10^9–1×10^{10} cells, respectively, in a single device.

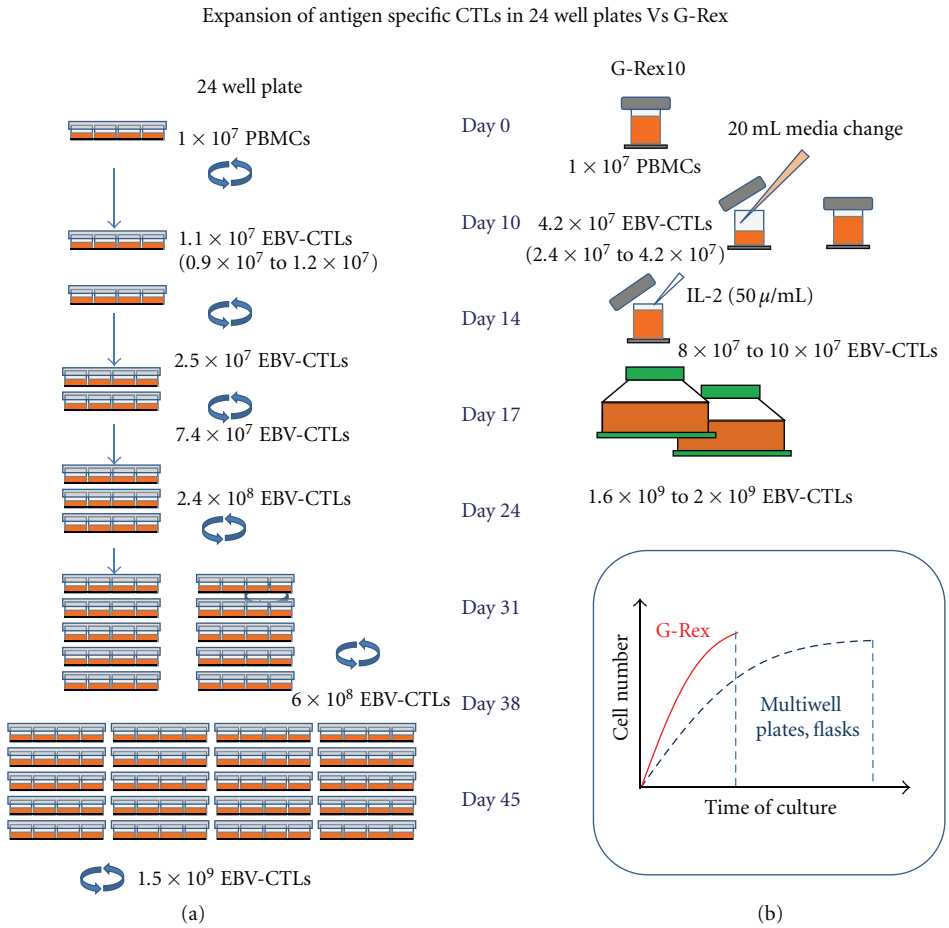


Figure 5: Optimization of antigen-specific CTL manufacture decreases the number of interventions while increasing the cell output. (a) illustrates the level of complexity associated with the generation of antigen-specific CTLs using conventional 24-well plates and the reduced number of interventions required when reproducing the same protocol using the G-Rex. (b) shows how the implementation of the G-Rex device decrease the *in vitro* culture time when compared with the conventional method.

8. Third-Party CTLs

These manufacturing improvements have allowed us to consider the use of virus-specific CTLs in the 3rd-party setting and recently we have developed a cell bank to facilitate this endeavor. Administration of this "off-the-shelf" product raises two potential concerns: (i) the risk of inducing GvHD by administering a partially HLA-mismatched CTL product and (ii) limited *in vivo* persistence, due to recipient alloreactivity directed against nonshared HLA antigens. Nevertheless a number of small studies have demonstrated the feasibility of this approach in the patients with EBV lymphoma arising after HSCT or solid organ transplant. Haque and colleagues used 3rd-party EBV-specific CTLs to treat PTLD after solid organ transplant or SCT and showed an encouraging response rate of 64% and 52% at 5 weeks and 6 months, respectively [33]. In this study the CTLs were selected by low-resolution typing and screened for high-level killing of donor EBV-LCLs and low-level killing of patient PHA blasts. The level of HLA matching ranged from 2/6 to 5/6 antigens, and there was a statistically significant trend towards a better outcome with closer matching at 6 months. Importantly, no patient developed GVHD after CTL administration. In another report two cord recipients with EBV lymphoma received closely matched EBV-specific T cells resulting in complete resolution of their lesions [34].

Currently we are evaluating the safety and potency of using "off-the-shelf" trivirus CTL for the treatment of CMV, adenovirus, or EBV infections in patients after HSCT with active infection and that do not respond to conventional therapy. Preliminary results in >35 recipients, most of whom had received alternative donor transplants, are encouraging, with minimal toxicity and >80% achieving complete or partial responses. If this trend continues, we will generate a larger CTL bank to cover as many racial groups as possible and progress to a phase II clinical trial where we can ask more specific questions regarding the persistence and function of the CTL *in vivo*. Such a study is dependent on the ability to produce large numbers of CTLs that maintain their specificity and functional activity and are not "exhausted" by excessive *in vitro* passaging, and this has become possible only recently with the advent of optimized culture protocols in the G-Rex cultureware that effectively supports CTL expansion.

9. Future Prospects

Manufacture optimization arises from constant and critical reflection on the different processes involved in the generation of a cellular product. The G-Rex culture device is just one example of manufacture optimization taking place at the CAGT. We have also recently simplified the process of virus-specific CTL generation by replacing viral vectors and live virus (previously used as antigen sources) with clinical grade plasmids and overlapping peptide libraries [35]. We have also discovered that certain combinations of enhancing and stimulatory cytokines support the efficient activation and expansion of both virus- and tumor-reactive CTLs, leading to the new GMP-compliant protocols that enable the rapid generation of high-quality cellular products. Although the manufacture optimization is a research phase that requires time, money, and effort, this is an investment and a prerequisite for the manufacturing success of a cell product. Ultimately, the final "value" of a cell product depends on the *in vivo* therapeutic efficacy; however, it is the manufacture process that either facilitates or restrains the evolution of such products from the boutique to the mainstream.

Abbreviations

Adv: Adenovirus
APC: Antigen presenting cells
CAR: Chimeric antigen receptor
CMV: Cytomegalovirus
CTL: Cytotoxic T lymphocytes
DLI: Donor lymphocyte infusions
EBV: Epstein-Barr virus
FBS: Fetal bovine serum
GVHD: Graft-versus-host disease
HSCT: Hematopoietic stem cell transplant
IL: Interleukin
LCL: Lymphoblastoid cell line
PACT: Production assistance for *cellular therapies*
PBMC: Peripheral blood mononuclear cells
TCR: T cell receptor.

Acknowledgments

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