

Cancer Specific CTL Expansion with ZYX Bioreactor

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Abstract

Objective: The use of therapeutic immune cell subsets is becoming an increasingly cost-effective and attractive strategy for the treatment of cancer. Nonetheless, the field of cell therapy is hampered by the inability to consistently generate and expand sufficient numbers of high quality cells from a majority of patients. To address this critical issue, the investigators developed a sophisticated bioreactor technology that maximizes metabolic support and minimizes the damaging shear-stress forces, automatically monitors functional correlates to allow cell harvest at peak functional capacity, and permits cell sorting *in situ* to minimize cell loss and microbial contamination. In current study, the established cancer-specific CTL expansion system will be tested for its potency of cancer cell killing both *in vitro* and *in vivo*.

Methods: To test the cell expansion efficiency of ZYX Bioreactor, the CD8⁺ T cells expanded in different culture systems were enumerated by flow cytometry and the Cancer-specific cytotoxic T lymphocyte (CTL) activity was measured for their cytokine production and against autologous tumor targets by CTL cytotoxicity assay *in vitro* and *in vivo* as well as Annexin V staining to detect low-level cytolytic activity. In the *in vivo* CTL assay, mouse cancer cell line was used as stimulators and targets for the expanded mouse CTL evaluation in BALB/c mice and human lung cancer cells and expanded human CTL were injected into immunodeficient mice for human CTL evaluation.

Results: In comparison of other cell culture systems, ZYX Bioreactor has significantly higher efficiency in the cancer specific CD8⁺ T cell expansion, and these CD8⁺ T cells expanded in ZYX Bioreactor exhibited higher specific CTL cytotoxicity in both *in vivo* and *in vitro* studies.

Conclusion: ZYX Bioreactor can provide the adequate metabolic support to the growing cancer-specific CD8⁺ CTLs and the proper condition for the stimulation of cancer cell-carried antigens to the CTLs.

Keywords: Cancer immunotherapy; Bioreactor; Cell culture and expansion; Animal experiment; Cellular immunology; Cytotoxic T lymphocyte; Adoptive transfer

Introduction

The elimination of cancer cells *in vivo* at least partially relies on the role of immunocytes. There exist a variety of innate and adaptive immune cell subsets that are currently in use for cell therapy of cancer [1-8]. Currently, CD8⁺ cytotoxic T lymphocytes (CTL), natural killer (NK) cells, dendritic cells (DC), mixed cell populations such as cytokine-induced killer (CIK) cells and tumor infiltrating lymphocytes (TIL), and genetically engineered immunocytes such as CAR-T cells have exhibited effective anti-cancer activity in clinical trials [9-15]. In addition, hematopoietic stem cell (HSC) transplantation has been successfully used for immune reconstitution of cancer patients and prevention of relapse of various cancers following intensive myeloablative chemotherapy. However, most clinical trials conducted using adoptive transfer of anti-cancer immunocytes has been largely unsuccessful. Since target cell lysis in both CTL and NK cell lytic assays correlates with the effector/target ratio at the log phase, increasing the cell number of CTL and NK cells in the therapy could reduce the cases of failure and increase the anti-cancer effectiveness of these cells. It is

of little doubt that all anti-cancer cell therapies experience this common challenge and will benefit from a more efficient expansion of higher quality cells.

Although a variety of tumor-specific mechanisms have been reported to mediate micro-environmental inhibitory effects on CTL activation [16-18], our recent studies have shown that irradiated or mitomycin C-treated cancer cells no longer inhibit CTL activity and the strength of the CTL inhibitory effect of unmanipulated cancer cells is positively correlated with the ratio of CTL cells/cancer cells. This is consistent with the findings of Ramakrishnan et al. in which chemotherapy was shown to enhance tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice [19]. Additionally, application of anti-CTLA-4, anti-CD137, and anti-PD1 has been shown to reverse inhibition of cancer cells to CTL killing [20,21]. These results further suggest that (1) increasing the effective CTL cell number can overcome or reduce the inhibitory effect of cancer cells on the CTL in cancer cell therapy, (2) *ex-vivo* treatment of cancer cells could eliminate their inhibitory effects on CTL activity, and (3) cancer cells treated in this manner might serve as better stimulators for cancer-specific CTL proliferation *ex vivo*. Hence an *in vitro* expansion protocol modified according to these criteria could be an effective strategy to generate enhanced numbers of high quality, tumor-specific CTL.

Recently, some cancer-specific peptide antigens has been identified and used to stimulate CTL, and CAR T cells are developed. They exhibit great potential and advantage. However, these methods can only be used in the patients with the cancer which specific antigen has been identified. In clinic, the cancer-specific antigens of most patients are often unknown. Therefore, using inactivated cancer cells as a stimulator to expand antigen-specific CTL is more feasible, more reliable and easier for clinical application. This is the reason for us to use the inactivated cancer cells to stimulate CTL in this study.

Currently, immunocytes are predominantly expanded in traditional static cultures such as flasks and plates. Bioreactors have also been tested for immunocyte expansion. In static culture, cells often suffer from poor metabolic support, especially when the cells reach higher densities. Tumor recognition and killing functions of the CTL cells are significantly diminished after expansion under such conditions. The application of bioreactor systems such as WAVE Bioreactor, rotation wall vessels, magnet stirring bioreactors, and micro-carrier bioreactors during expansion of immunocytes can significantly improve metabolic support for expanding cells by maintaining suspension status. However, continuous cell re-suspension greatly diminishes the stimulation of cancer cells to CTL by reducing the opportunity of cell contacts and creates strong shear-stress forces on the cells and results in cell damage and death by accelerating apoptotic processes. These bioreactors definitely do not provide the basic condition for cancer cells to stimulate antigen-specific T cells. Therefore, there is a clear need for a bioreactor to provide cells with ideal metabolic support with concomitant minimization of shear-stress and ensure that effector cells and stimulator cells can make the sufficient contacts.

The ZYX Bioreactor (ZYX Btr) allows cells to be alternatively cultured under both static and kinetic conditions, allowing cells evenly distributed on the bottom of culture chamber or along the surface of the agitators during the change from the kinetic to the static state so that cells receive maximum metabolic support and minimal shear-stress. Such conditions are important during the activation and expansion of immunocytes such as CD8⁺ CTL in which contact between the effector and stimulator cells is essential.

The expansion of anti-cancer CD8⁺ CTL requires the stimulation of the TCR by means of MHC class I complex with specific peptide antigens. Since most cancer-specific antigens for patients in clinics are unknown, inactivated cancer cells from patients' cancer tissue provide the optimal potential peptide antigens for specific CTL stimulation. For close contact between CTL effectors and tumor stimulators, a suitable proportion of CTL and cancer cells at high density are required. Under such conditions, traditional static culture cannot provide adequate metabolic support for the rapid growth of high density CTL. Kinetic cultures with commercially available bioreactors maintain cells in permanent suspension, minimizing opportunities for meaningful cell to cell contact. The critical disadvantage of bioreactors available in the current market is the lack of a mechanism allowing cells to be evenly distributed, resulting in cell accumulation in the cell culture container when kinetic culture ceases. Cell accumulation significantly inhibits CTL growth, yet kinetic bioreactors available in market do not stop running during cell culture process. Therefore, the cells have no opportunity to make contact. In contrast, the ZYX Btr provides alternative culture between static and kinetic modes allowing CTL to receive the optimal metabolic support and the proper stimulation from inactive cancer cells by effective cell contact. Additionally, the ZYX Btr has a function that permits cell sorting and cell culture in the same chamber to allow separation of CTL from

tumor cells in a highly efficient fashion and also almost entirely eliminate contamination risks. The combination of cell sorting and cell culture in a single chamber also benefits all lymphocyte expansion and can upgrade a variety of mixed cell expansions. For example, CD8⁺ CTLs can be isolated from total TILs. The ZYX Btr also has many other advantages that can enhance all types of anti-cancer immunocyte expansion (for more details about the ZYX Btr structure and working principles, see our published patent information: PCT/US2012/000182).

ZYX Btr has been tested for the expansion of hematopoietic stem cells and it has exhibited many advantages over other cell culture methods by reducing the non-specific differentiation and providing more effective metabolic support. In current studies, ZYX Btr is further investigated for its potency in expansion of anti-cancer specific CTLs.

Methods

The evaluation of expanded CTL consists of two main aspects, quantity and quality. For its quantity evaluation, the expanded cell number will be counted, and for its quality evaluation, the specific-killing function of expanded CTL to target cells, which includes classic CTL assays and Annexin V binding assay, and IFN- γ production level of expanded cells was accessed. First, the expanding CTLs were counted and characterized using flow cytometry to enumerate cells with a characteristic marker expression (CD8) as per established protocols [22-24]. As our routine procedure, all cells were also counted by Trypan Blue staining for live and died cells. Secondly, expanded CTLs were functionally characterized to determine the target cell killing potential. CTL lytic assays [22-25] were used for *in vitro* target cell killing functional evaluation and defined cancer animal models were used for functional evaluation of cancer cell killing *in vivo*. In the *in vivo* experiments of this study, BALB/c mice received cancer cell lines and mouse CTL injection, while NOD/SCID mice [26-29] were inoculated with cancer cells from human patients after which CTLs from the same individual were used for adoptive transfer.

Mice and grouping

BALB/c (H-2^d) mice (4 months) and NOD/SCID mice (4 weeks) were purchased from Charles River Labs. These animals were housed in specific pathogen-free environment and cages were covered with barrier filters with sentinel cages in Lamina Hoods (Lab products Inc.). The Zyxell Animal Protocol and Research Committee approved the use of animals according to principles expressed in the National Institutes of Health, USPHS, *Guide for the Care and Use of Laboratory Animals*.

In the *in vivo* mouse CTL study, each BALB/c mice (male, 4 in each group) received 0.2×10^6 or 1×10^6 Renca cells by subcutaneous injection and 2×10^6 above expanded CD8⁺ T cells by intravenous injection. Tumor size were measured double-blinded each week for 6 weeks. In the *in vivo* human CTL study, human lung cancer cells were used as stimulator cells (irradiated at 20 Gy) for CTL expansion and as target cells for the subcutaneous inoculation, and the T cells were from the peripheral blood of the same patient. Irradiated (3.5 Gy) NOD/ SKID mice (6 in each group) were used as the recipients.

Enumeration of cells: Total cells were counted using two standard methods: trypan blue dead cell exclusion and flow cytometry PI staining. The enumeration of CD8⁺ CTLs, CD3⁺ T cells, CD33⁺ cells, CD20⁺ cells and Annexin V⁺ cells was conducted using flow cytometry, and cell markers were stained by means of established standard protocols [22-25]. All staining reagents were from BD Pharmingen.

CTL expansion and CTL assay: Commercially available human cancer cells and the autologous mononuclear (Buffy coat cells from peripheral blood, ZYX Biotech Company, Texas, USA) were used for human CTL evaluation. Renca cells (mouse cancer cell line from ATCC) and splenocytes from Renca cell-primed BALB/c mice were used for mouse CTL evaluation. Mononuclear cells were isolated and seeded in a ZYX Btr (ZYX Biotech Company, Texas, USA) cell culture chamber and 6-well plates at 10^6 /ml and cultured for 6 days with an RPMI1640 containing FBS or mouse serum, 8 ng/mL IL-2 and 10 ng/mL IL-7 (4 cytokines for either human or mouse, all from PeproTech, Rocky Hill, NJ, USA), and irradiated cancer cells (seeding density determined by surface area, 80% confluent). Cancer cells were used as stimulators and target cells and mononuclear cells as effectors. Established protocols [22-25] with slight modification were used in this study. In brief, (1) cancer cells were grown and enriched *in vitro*. (2) Some of these cells were treated with mitomycin C and cultured with the mononuclear cells from the same patient in static culture, continuous suspension bioreactors and ZYX Bioreactors with IL-2 and IL-7 for 6 days as determined by the computer program in the ZYX Btr control system. Cells in static culture and continuous suspension bioreactors (controls) were harvested on the same day. (3) CD8⁺ cell isolation was accomplished using the cell sorting program in the ZYX Btr or using a Miltenyi cell separation device for the controls. Additional controls were consist of con A-treated CD8⁺ cells, unstimulated CD8⁺ CTL and/or the CD8⁺ cells stimulated with different tumor types. (4) *In vitro* CTL assay were performed as previously described [22-25] in which target cells were the cancer cells. Different cancer cells will also be used as control targets. Based on our preliminary studies, E:T ratios of 8:1, 16:1, and 32:1 were used for setting the cell lysis tests. LDH Cytotoxicity detection Kit (Clontech, Cat#630117) was used for the CTL Cytotoxicity analysis by following the Manufacturer's instructions with a slight modification as in our previous description [23-25], except that the cell lysis occurred in the culture with the T cells not stimulated by cancer cells as background was subtracted. In more detail, 2x, 4x, or 8x 10^5 /well effector cells were added into 25,000/well target cells in triplicate and incubated for 4 hrs. Supernatants were then harvested for LDH detection. CTL activity (% lysis) was calculated using the formula (Test cell mix-Effector control-spontaneous release)/(maximum release-spontaneous release). (5) For the *in vivo* CTL assay, cancer cells were enriched and injected into mice as described in (1) and (2); cancer-specific CTL were prepared as described in (3) and (4) and were adoptively transferred into mice in the tail vein on day 3 following cancer cell injection. On the day 21, mice were euthanized, and the numbers of lung metastasis were counted and subcutaneous cancer nodes were measured in a double-blinded fashion. The animal experiments were performed in the animal facility of Zyxxell Inc.

Test early stage apoptotic cells: Annexin V banding assay were done with established protocols [24]. It was assessed by the percentage of Annexin V positive cells/CD3-CD20-CD33- cells, in which the percentage of Annexin V positive cells for the culture with the T cells not stimulated by cancer cells as background was subtracted.

Interferon gamma (IFN- γ) examination: TFN- γ is the critical cytokine in regulating CTL killing function. ELISA (R&D) was used for the IFN- γ detection of the supernatant from CTL expanding culture. Flow Cytometry will be used for intracellular IFN- γ analysis, which was analyzed by the percentage of IFN- γ and CD3 double positive/CD3 positive cells. These assays were done by following the manufacturers' instruction as described before [22-25].

Comparison with other cell expansion systems: Since there is no bioreactor that combines static and kinetic cultures and combines the cell culture and cell sorting (see our published patent information: PCT/US2012/00086), we selected two kinetic bioreactor systems, which are commercially available, and the traditional static culture, which has most commonly been applied in CTL expansion, to be control groups (6 in each group) in our comparison studies as described in the "CTL expansion and CTL assay" section.

Data analysis: ANOVA was used to compare the CTL cell number and the % of cell lysis between different groups. Simple and polynomial regression analyses were used for the establishment of the regression functions for the relationship between cell fold-expansion and the ratio of cell-fold expansion versus the % of cell lysis. SAS and SAS Statview were used for statistical analysis.

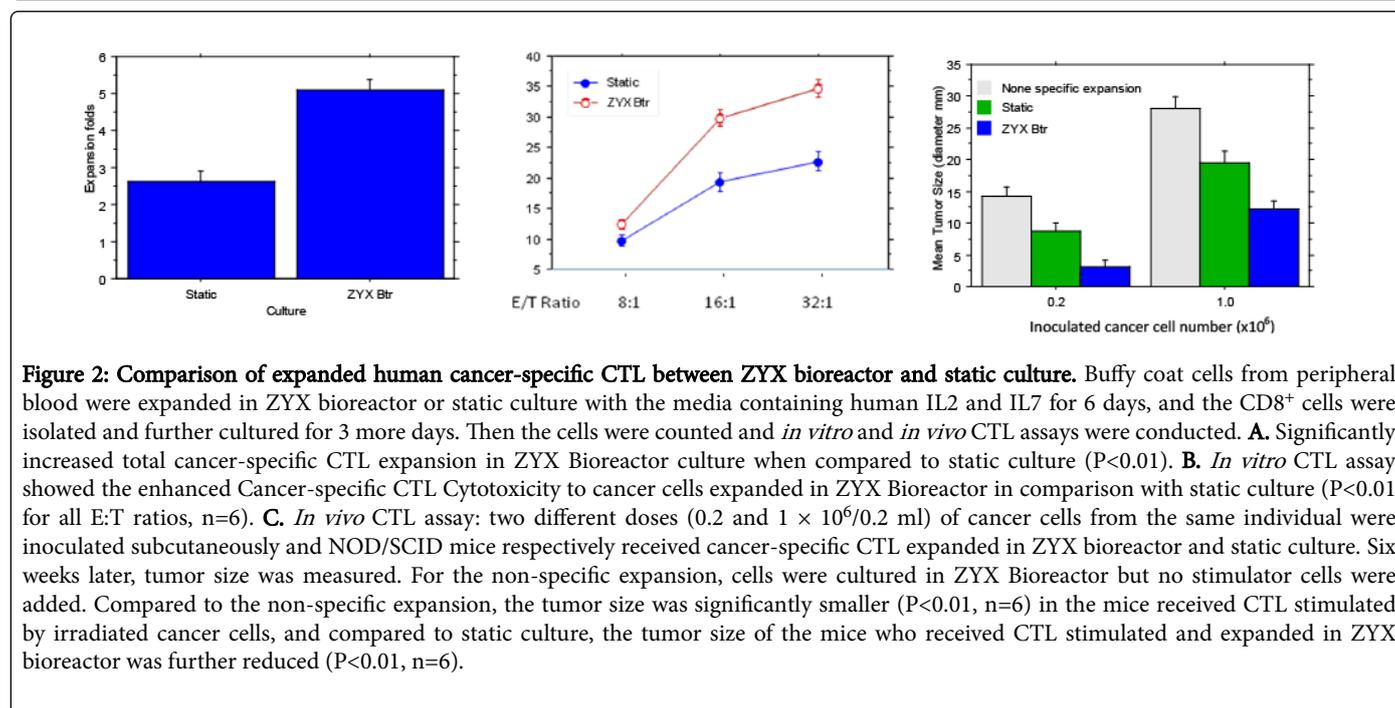
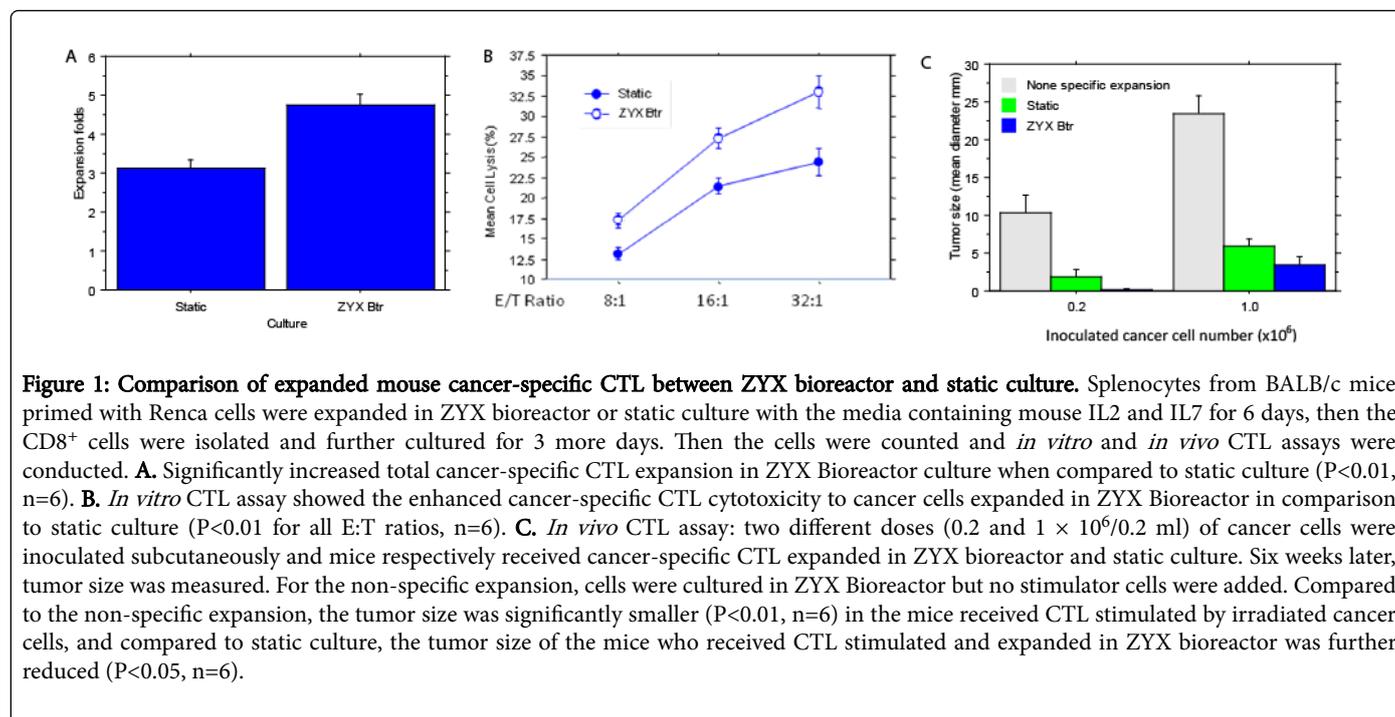
Results

Improvement of mouse cancer cell-specific CD8⁺ cytotoxic T lymphocyte expansion by ZYX Btr

Splenocytes from cancer cell-primed BALB/c mice were isolated and seeded in a ZYX Btr cell culture chamber and 6-well plates at 10^6 /ml and cultured for 6 days with an RPMI containing mouse serum, IL-2, IL-7, and irradiated (20 Gy) cancer cells (Renca cells, seeding density determined by surface area, 80% confluent). On day 6, suspension cells in the ZYX Btr were processed for CD8⁺ cell isolation using a positive selection program and then cultured with the same medium for 3 more days. CD8⁺ cells in a static culture were isolated using a Miltenyi device and then placed back in plate to culture with the same medium for 3 more days. Following the expansion, the CD8⁺ cells were counted by flowcytometry (Figure 1a), and *in vitro* (Figure 1b) and *in vivo* (Figure 1c), CTL assays were conducted. The figure 1a shows that the number of CTLs expanded in ZYX Btr was significantly higher than that in static culture. The figure 1b indicates that when the effector cells were stimulated in the ZYX Btr, the CTL fold-expansion folds overall lytic activity were significantly higher than those in the static culture ($p < 0.05$). In the *in vitro* CTL assay (Figure 1c), the growth of tumors of the mice received CTLs expanded in ZYX Btr was suppressed greatly when compared to the static culture.

Improvement of human cancer cell-specific CD8⁺ cytotoxic T lymphocyte expansion by ZYX Btr

Peripheral blood from a patient with small cell lung cancer was used for the CTL expansion. On the day 6 of the buffy coat cell culture with the irradiated (20 Gy) cancer cells from the same individual, the CD8⁺ cells were isolated and further expanded in the media containing human IL2 and IL7. The procedure of cell counting and CTL assays were the same as for the mouse CTL assay except that the stimulator cells, effector cells and target cells are human cells. For the *in vivo* CTL assay, the recipient mice were the irradiated NOD/SCID mice. Similar to above mouse CTL assay, ZYX Btr also significantly increased the expanded human antigen-specific CTLs in both their number (Figure 2a) and their cancer cell killing function (Figure 2b), in comparison with the static culture. Consistent to the *in vitro* assay and mouse *in vivo* experiments, the average size of the tumors of the mice that receive the CTLs expanded in ZYX Btr was significantly smaller than that of the controls. These results demonstrate that ZYX Btr provides better growth condition to human antigen-specific CTL both for their proliferation and for their cytotoxicity.



Increased Annexin V expression on the target cells attached by *in vitro* expanded human antigen-specific CTL

Annexin V positive indicates early stage of cell apoptosis. After the *in vitro* CTL assay, cells were examined by Annexin V binding assay for CD8 negative (CD8⁻) cells. The T cells without stimulators and the T cells expanded in static culture were served as controls. The results exhibited in figure 3 showed that the more target cells were positive for Annexin V in the group with the T cells expanded in ZYX Btr

compared to other groups, suggesting these expanded CTLs function by accelerating the apoptosis of target cells.

Elevated Interferon- γ level in the CTL expanded in ZYX Btr in comparison to the static culture

- INF- γ level was higher in the cell culture media of ZYX Btr than that in static culture.

- INF- γ level in the culture media was assessed by ELISA. As shown in figure 4, the culture with ZYX Btr contains the highest level of INF- γ .
- Intracellular INF- γ expression was higher in the CTL expanded in ZYX Btr than that in static culture.

Cells were also double-stained for CD8-FITC and Intracellular INF- γ -PE. As shown in figure 5, the CD8 and INF- γ double positive/CD8 positive ratios are at similar level among all groups, but the PE mean intensity for ZYX Btr group is significantly higher than controls.

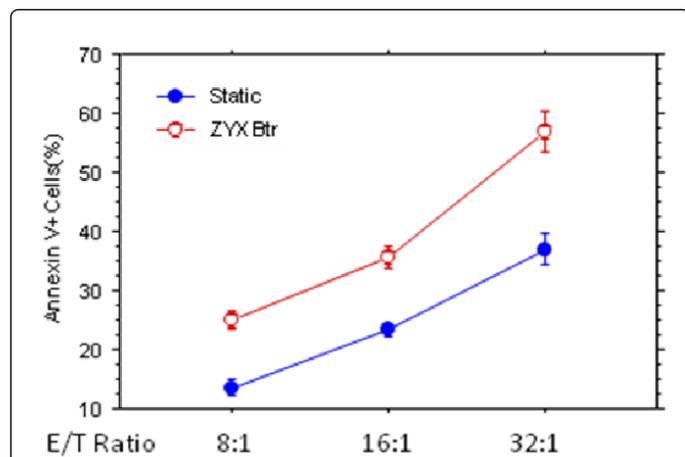


Figure 3: CTL expanded in ZYX Bioreactor leads more Annexin V expression of target cells. Annexin V banding assay were conducted with flowcytometry. It was assessed by the percentage of Annexin V positive cells/CD3-CD20-CD33- cells, in which the apoptosis occurred in the culture with the T cells not stimulated by cancer cells as background was subtracted. The CTL expanded in ZYX Bioreactor induced more Annexin V expression of target cells in comparison with static culture at all E:T ratios ($P < 0.01$, $n = 6$).

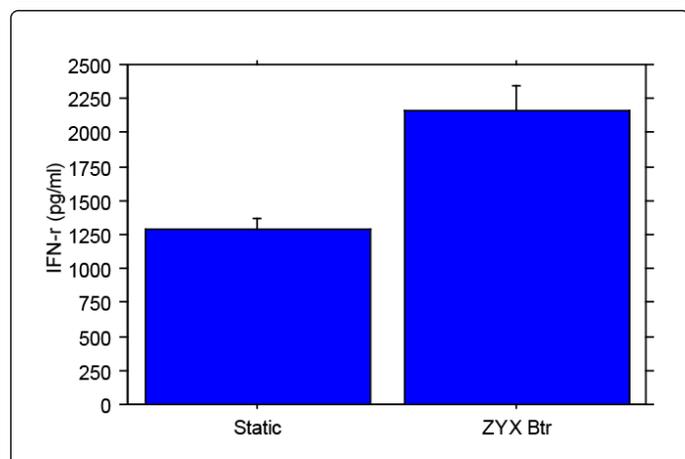


Figure 4: Human CTL culture media in ZYX Bioreactor contains more Interferon- γ (IFN- γ). Buffy coat cells from peripheral blood of a patient with lung cancer was Cell expansion media collected on day 6 was used for IFN- γ analysis with ELISA. IFN- γ in the culture of ZYX Bioreactor has significantly higher level in comparison with static culture ($P < 0.01$, $n = 6$).

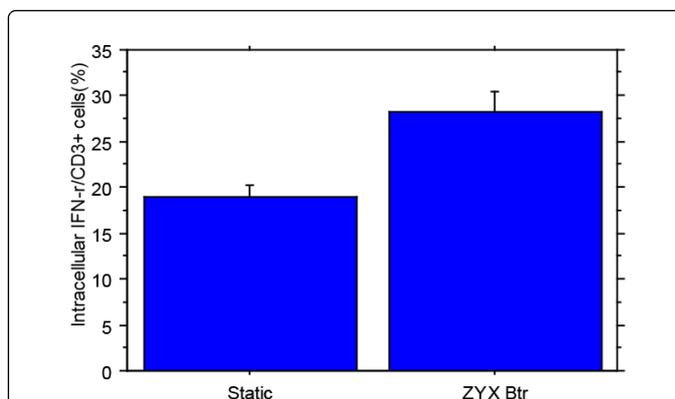


Figure 5: More Interferon- γ (IFN- γ) was expressed in human T lymphocytes expanded in ZYX Bioreactor. Cells were collected on day 6 and stained with IFN- γ -FITC and CD3-PE. The percentage of IFN- γ and CD3 double positive/ CD3 positive cells was obtained from flow cytometry analysis. The significantly more T cells in ZYX Bioreactor culture expressed INF- γ than static culture ($P < 0.01$, $n = 6$).

Discussion

Several bioreactor systems currently exist, and some have been considered as candidates for immunocyte expansion [30-39]. However, these systems all have specific disadvantages. One of these cell culture systems is prepared using gel or gel-like cell support materials such as hydrogel [31-34] to maintain a 3D growth environment for cells. These materials are non-physiologic; their static nature impedes the growth of suspension cells such as lymphocytes and HSCs. Rotation-based 3D culture systems [35,36] do not require gel-like materials to support cells. Their slow vertical rotation maintains the suspension of cells or the bead-attached cells in 3D growth states. However, the rotation-based 3D culture leads to cell accumulation at the curved bottom when the rotation ceases. This accumulation prevents normal cell growth. Moreover, rotation systems maintain high shear-stress forces, which result in significant immunocyte damage. Cells in suspension state also seldom have the opportunity to make contact, including contact between effector cells and stimulator cells in CTL expansions. Other kinetic cell culture bioreactors can exert even higher levels of shear-stress on cells in suspension and also cannot avoid cell accumulation and aggregation when the suspension motion ceases. Definitely, the cell accumulation and aggregation have harmful effects on cell expansion, while continuous stirring maintains constant shear-stress [40-44]. Hence, these kinds of bioreactors are suboptimal for CTL expansion.

When hydrogel technologies are combined with scaffolds or rotational wall technologies, the disadvantages common to kinetic bioreactors are recapitulated [30-34,45-49]. The ZYX bioreactor allows alternating culture between static and kinetic states so that cells can be evenly distributed at the bottom or the surfaces of the agitators during the change from kinetic to static states. This feature permits maximum metabolic support, minimal shear-stress forces, and allows opportunity for cell to cell contact.

Our previous studies demonstrated that CTLs gradually losing their killing function after *in vitro* culture. The killing capacity per million effector cells on culture days 12, 18, 24, and 30 dropped by

approximately 1/3, 1/2, 7/8, and 19/20, respectively, in comparison with day 6 in spite of continuous increases in cell number. It was also discovered that total CTL killing capacity has a peak time period during the expansion and decreases very quickly after the peak time period. This suggests that expanded CTL or NK cells should be harvested at or near the peak time point for maximum killing effectiveness. Our studies also revealed a correlation between the cell expansion fold and the cell killing capacity; therefore, the peak time can be estimated according to the cell density. Figure 6 illustrates the relationship between cell density and the total cytotoxic killing effectiveness. These discoveries allow the ZYX bioreactor to automatically determine the optimal cell culture duration. The bioreactor monitors cell growth, estimates cell function at the peak time, and terminates the culture at that peak time to maximize the efficacy of the expanded immunocytes.

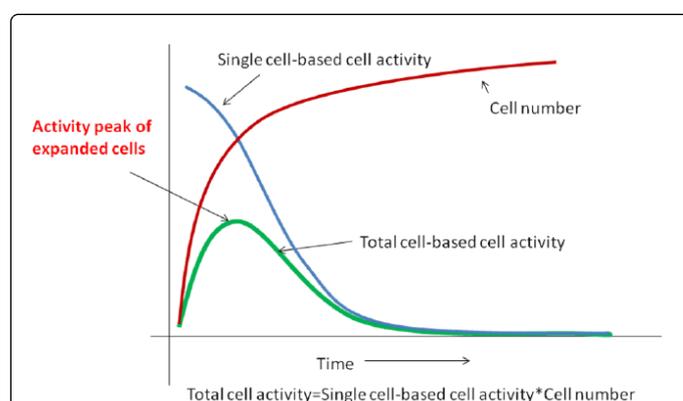


Figure 6: Relationship between cell activity and cell number in expanding cells. This is a strategy for ZYX Btr to harvest cells at their peak activity point. Many cells gradually lose their functions or activities (eg. CTL reduces its cytotoxicity, hematopoietic stem cell loses its engraftment potential) during their *in vitro* expansion.

At early stage of the expansion, the cell number increases faster than the activity decreases, which results in total activity increases. Afterwards, the individual cell-based activity drops very fast and leads the total activity decreases. Therefore, the best cell harvest time should be at the time point right before the total activity decreases.

The ZYX bioreactor has a perpendicular re-suspension system controlled by a computer, allowing for the vertical re-suspension of cells by means of magnetically-controlled agitators in conjunction with buoyant and gravitational forces. This process creates even cell distribution on the bottom of the cell culture container and/or the surface of the agitators when agitation ceases. This allows cells to be cultured in alternative states (i.e., between static and kinetic states) so that cells can grow in an optimal metabolic environment with minimal shear-stress. Also, ZYX Btr allows the cell sorting and cell culture to be conducted in the same container in which cell transfer between containers is not needed and so it greatly reduces the cell loss and avoids the possible contamination during the cell transfer between the two processes and regular cell sorting process.

With these advantages, ZYX Btr has been successfully used for hematopoietic stem cell expansion, in which the non-specific differentiation was significantly reduced and cell engraftment potency was greatly enhanced. In the current studies, ZYX Btr was further

tested for its potential in the expansion of cancer-specific CTL. The results in the mouse CTL expansion showed the CTL expanded in ZYX Btr did not only exhibit higher cell density but also stronger specific cytotoxicity to cancer cells both *in vitro* and *in vivo*. Although mouse *in vivo* CTL assays can reveal the cytotoxic killing function of CD8⁺ t cells in the body of same species, the expanded human T cells need to be further examined for their function before they can be used to clinical trial. In this study, a classic animal model [26-29] for human cell transplantation was used to evaluate the cancer killing function of the expanded cells. In addition to significantly increased cell number and *in vitro* cytotoxicity to cancer cells, the human CTL expanded in ZYX Btr more effectively inhibit the *in vivo* growth of lung cancer cells obtained from the same individual in comparison with the static culture. These data indicates that ZYX Btr can provide better condition for the CTL expansion. In our experiments, the cultures without cancer cell stimulation were also observed as controls. However, cells without cancer cell stimulation exhibited the least (if any) cancer cell killing function, suggesting that the specific stimulation to CTL play a critical role in the effective antigen-specific CTL expansion. Considering the controls with continuous cell suspension showed the similar level (data not shown) of tumor growth inhibition of cells without specific stimulation and similar to the control without cell adoptive transfer (data not shown), the cell contact between effectors and stimulators is required for inducing and keeping specific cytotoxicity of CTL.

The of target cell lysis related to CTL cytotoxic cell killing involves a mechanism of apoptosis [24]. Annexin V is a cell membrane marker of apoptosis which appears at the early stage of apoptosis. In order to confirm that the cell lysis caused by expanded CTL is still mediated by apoptosis, Annexin V on the target cells was examined. Consistent to the CTL assays, the expression of the Annexin V on the target cells of the CTL expanded in ZYX Btr were enhanced. It suggests that the CTL expanded in ZYX Btr were still functioning by triggering out the apoptosis of target cells.

INF- γ is an up-regulator of Th1 immune response and correlated to CTL activity [23-25]. Therefore, INF- γ level can also reflect the quality of the *in vitro* expanded cells. In our current studies, the intracellular INF- γ level and INF- γ concentration in the culture media are all higher in the CTL expanded in ZYX Btr than in controls, suggesting that ZYX Btr provided a more suitable condition for cells to produce and secrete INF- γ .

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