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Single-Pass, Closed-System Rapid Expansion of Lymphocyte Cultures for Adoptive Cell Therapy

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Abstract

Adoptive cell therapy (ACT) for metastatic melanoma involves the *ex vivo* expansion and re-infusion of tumor infiltrating lymphocytes (TIL) obtained from resected specimens. With an overall objective response rate of fifty-six percent, this T-cell immunotherapy provides an appealing alternative to other therapies, including conventional therapies with lower response rates. However, there are significant regulatory and logistical concerns associated with the *ex vivo* activation and large scale expansion of these cells. The best current practice uses a rapid expansion protocol (REP) consisting of an *ex vivo* process that occurs in tissue culture flasks (T-flasks) and gas-permeable bags, utilizes OKT3 (anti-CD3 monoclonal antibody), recombinant human interleukin-2, and irradiated peripheral blood mononuclear cells to initiate rapid lymphocyte growth. A major limitation to the widespread delivery of therapy to large numbers of melanoma patients is the open system in which a REP is initiated. To address this problem, we have investigated the initiation, expansion and harvest at clinical scale of TIL in a closed-system continuous perfusion bioreactor. Each cell product met all safety criteria for patient treatment and by head-to-head comparison had a similar potency and phenotype as cells grown in control T-flasks and gas-permeable bags. However, the currently available bioreactor cassettes were limited in the total cell numbers that could be generated. This bioreactor may simplify the process of the rapid expansion of TIL under stringent regulatory conditions thereby enabling other institutions to pursue this form of ACT.

Keywords

Adoptive cell therapy; tumor infiltrating lymphocyte; rapid expansion; closed system bioreactor; rhIL-2

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1. Introduction

Adoptive cell therapy (ACT) involves the *ex vivo* isolation and expansion of antigen-specific lymphocytes for the purpose of autologous infusion. The applications of this therapy for cancer have included the treatment of Epstein-Barr virus (EBV) induced malignancies and metastatic melanoma.(Rosenberg et al., 2008) Experimental protocols conducted at the National Cancer Institute-Surgery Branch (NCI-SB) have demonstrated the feasibility of inducing tumor regression in patients with metastatic melanoma following the infusion of tumor infiltrating lymphocytes (TIL). After isolation of TIL from resected metastatic lesions, rapid expansion in culture and re-administration to late stage melanoma patients, these lymphocytes, when administered following a short course of lymphodepleting chemotherapy, achieved objective responses by RECIST criteria (Response Evaluation Criteria in Solid Tumors) in 52 out of 93 patients (56%).(Dudley et al., 2008)

Patients enrolled in initial clinical trials received an average dose of 6.0×10^{10} TIL per infusion. In order to generate this sizable cell product over a short period of time, TIL underwent a rapid expansion *ex vivo*, using anti-CD3 antibody, exogenous interleukin-2, and irradiated peripheral blood mononuclear “feeder” cells. This rapid expansion process (REP) was started in T-flasks and completed in gas-permeable (3-liter) bags. The cell expansion, viability, and performance achieved with current REP technology have been excellent for phase I/II developmental studies, and have allowed flexibility in the establishment of standard operating procedures. However, these current REP protocols present several limitations to the eventual widespread implementation of adoptive cell therapy. First, the REP in flasks/gas-permeable bags is an “open” system that necessitates multiple exposures of the cell product to the external environment for feeding, sampling, manipulation, and the transfer of TIL from flasks to bags. Such frequent manipulation allows for the possible contamination of the cells. Secondly, the use of numerous containers introduces the possibility of vessel-to-vessel variability. Third, this system is labor intensive requiring the devoted attention of skilled laboratory technicians.

Development of an optimal method of rapid expansion for widespread clinical use would involve a “closed” system thereby minimizing the chance of in-process contamination while maintaining comparable cell yields and requiring minimum expertise and labor. Several bioprocessors such as the Wave® and hollow-fiber bioreactors have been tried (Hami et al., 2004;Levine, 2008). These systems have proven useful for some clinical applications with lymphocytes, but none that involve the initiation of large scale *in vitro* expansions with antigen presenting cells or feeder cells. We report here our investigations of a bioreactor developed at Aastrom Biosciences (Ann Arbor, MI) (Koller et al., 1993;Koller et al., 1998;Guardino et al., 2006). It has been previously employed in the *ex vivo* expansion of umbilical cord blood cells, bone marrow stem cells, and for clinical production of dendritic cell vaccines. These cell products have then been used to treat patients with chronic myelogenous leukemia, bone marrow suppression following high-dose chemotherapy, and for dendritic cell-based immunotherapy of multiple myeloma and other cancers.

We report here the initiation, completion, and preliminary optimization of the rapid expansion of multiple TIL samples in this closed-system perfusion bioreactor. In addition, we evaluate the antigen-specificity, potency, viability, sterility, and phenotype of TIL produced in the bioreactor system when compared to static culture conditions in T-flasks and gas-permeable culture bags.

2. Materials and Methods

2.1 Tumor Infiltrating Lymphocytes (TIL)

The TIL cultures used for this set of experiments were generated by employing a variety of techniques that have been described previously(Dudley et al., 2003). Briefly, TIL cultures were created following the overnight enzymatic digestion of a tumor specimen, by physical disaggregation using a Medimachine (Becton-Dickenson) with subsequent lymphocyte enrichment on a ficoll-step gradient, or from 1–2 mm tumor fragments. The age of the all TIL cultures expanded and used in this set of experiments varied from twelve to twenty-five days, determined as the time from initial culture to the start of the rapid expansion. TIL culture media consisted of a mix of 50% AIMV (Gibco, Grand Island, NY) and 50 % complete medium (CM) supplemented with 6000IU/mL IL-2. CM is comprised of RPMI 1640 (Biowhittaker, Walkersville, MD), 25 mmol/L HEPES pH 7.2, 100 U/mL penicillin, gentamicin 10Ig/mL, 5.5 $\times 10^{-5}$ M β-mercaptoethanol, ciprofloxacin 10 μg/mL, supplemented with 10% human AB serum.

2.2 Closed-system Bioreactor

The closed-system parallel plate bioreactor used for this set of experiments is a research and development component set produced as a derivative of a clinical product manufacturing device produced by Aastrom Biosciences, Inc (Ann Arbor, MI). It consists of a disk-shaped biochamber that has a liquid compartment and a gas chamber separated by a gas permeable, but liquid impermeable membrane (Figure 1). At the base of the liquid chamber is a round tissue culture-treated plastic disc that makes up the cell bed. This disc has a surface area of 850 cm^2 and the total capacity of the liquid chamber is 300 mL. Media is perfused into the cassette through a center port while gas transport occurs via diffusion through the aforementioned gas permeable membrane. This “decoupling” of gas transport from media perfusion allows for the slower, laminar flow of media over the cell bed and permits the culturing of non-adherent cells (i.e. lymphocytes) in this device. This operating mode is unique to the Aastrom bioreactor and is referred to as “single-pass perfusion”. Spent media drains through a series of holes at the perimeter of the disc and can be sampled for testing prior to collection in the waste reservoir. Waste medium eventually collects in a two-liter reservoir under the cell bed compartment and will then drain into a waste bag when that reservoir becomes full.

2.3 Priming and Inoculation

Each biochamber is contained within a disposable cell cassette which inserts into a robot-processor or “platform” that is designed for the automated completion of two initial steps: priming and inoculation. Priming is done 2.5 to 24 hours before inoculation by the addition of roughly 300 mL of culture media. Priming allows for the conditioning of the cell bed as well as the degassing and equilibration of the medium. During the time interval between priming and inoculation, each cassette resides in an incubator at 37°C in 20% O_2 and 6% CO_2 with an air pump and bubble humidifier attached.

Inoculation involves the injection of a combination of irradiated (50Gy), peripheral blood mononuclear cells “feeders” (1×10^9/cassette) and TIL (5×10^6, 1.5×10^7, or 2.0×10^7) plus 300 μL of rhIL-2 (6000IU/mL, final concentration; Chiron Corp., Emeryville, CA) and 9 μL of OKT3 (1mg/mL anti-CD3 antibody, 30ηg/mL final concentration; Ortho Biotech, Bridgewater, NJ) into the center injection port of each cassette. This mixture is generally 20–30 mL and is injected by syringe into the bioreactor. Following inoculation, each cassette is placed individually on the processor - a proprietary robotic platform - and the cells are distributed evenly across the cell bed using a series of programmed valve actuations and movements, followed by purging of the remaining air from the cassette. Each inoculated cassette is then returned to the incubator.

2.4 Perfusion

For 48 hours after inoculation, the cassettes reside in an incubator at 37°C in 20% O_2 and 6% CO_2 with a separate air pump and bubble humidifier attached to each. At the end of day 2, tubing is run from a media bag containing AIM V media, 1% or 5% human serum, 100U/mL penicillin, 2mM L-Glutamine (MediaTech, Manassas, VA), 10ug/mL ciprofloxacin (Bedford Laboratories, Bedford, OH), 6000 IU/mL rhIL-2, and 1.25 μg/mL amphotericin B (Xgen Pharmaceuticals, Big Flats, NY), through the gasket in the side of the incubator and sterilely welded (Terumo, Somerset, NJ) to the media perfusion line of each cassette. Between the media source and the cassettes, peristaltic pumps (Watson-Marlow Bredel, Wilmington, MA) are used to maintain the constant flow of media. Per an established protocol, each roller pump is started at three RPM providing a medium exchange rate of 5.8 mL per hour. Starting on day 3, spent media is removed via the sampling port for lactate analysis. The lactate concentration in effluent medium was quantified by enzymatic determination with comparison to a lactate standard according to manufacturer's instructions (Trinity Biotech, Berkeley Heights, NJ). Absorption measurements (O.D. 540 nm) were determined using a Beckman DU640 spectrophotometer (Beckman Coulter, Inc. Fullerton, CA). The rates on the peristaltic pumps were adjusted to achieve a ramped perfusion rate of 5.8–70 mL per hour with a goal of maintaining the lactate levels between 0.5–1 mg/mL. The perfusion rate adjustment was dependent on the growth rate and cell density of the lymphocytes in the bioreactor and was adjusted for each independent batch.

At the end of each experiment, the total lactate produced for each cassette was calculated by summing the daily lactate production values. To calculate the daily production of lactate in each cassette, we first multiplied the total volume in the cassette (300 mL) by that day's measured lactate level (mg/mL), and then subtracted from this value the product of the estimated volume of media still in the cassette from the previous day and the previous day's lactate measurement. An example of the formula is below: Lactate produced in bioreactor between third and fourth day = 300*(day 4 lactate measurement)−(1-% media exchange between third and fourth day/100)*300*(day 3 lactate measurement)

The % media exchange from one day to the next was estimated based on the daily perfusion rate for each cassette.

2.5 Harvesting

For all but three experiments, the duration of rapid expansion in each cassette was carried out for 14 days. On the day of harvest, the media line from each cassette was disconnected and the cassette was removed from the incubator and placed on the processor. A 1 L bag of Isolyte solution (BBraun, Bethlehem, PA) was suspended from a tubing holder for the purpose of rinsing the cells during the harvest sequence. Following the completion of this step, the washed cells were collected directly into a harvest bag attached to the cassette using the automated harvesting sequence programmed into the processor.

2.6 Rapid Expansion in T-flasks/gas-permeable bags

In parallel with rapid expansion in the closed-system bioreactor, TIL were expanded using techniques that have been described previously (Dudley et al., 2003;Riddell and Greenberg, 1990;Tran et al., 2008). Briefly, the same allogenic, irradiated feeder cells (2×10^8), OKT3 antibody (30 η g/mL), rhIL-2 (6000IU/mL), CM (75 mL), AIM V (75 mL), and TIL (1×10^6) were combined, mixed, and aliquoted into one 175 cm^2 tissue culture flask. This flask was then incubated upright at 37°C in 6% CO_2. On day 5, half the media was replaced with a 1:1 mixture of CM/AIM V containing 6000IU/mL rhIL-2. On approximately day 7, the cells in the flask were transferred to Baxter 3-L culture bags. Media was added to these bags as

needed to maintain the cell density at around 0.5×10^6 cells/mL. The TIL in each bag was harvested on the same day as the cassettes.

2.7 Sterility and Cytopathology

Following the harvesting of cells from the biochamber, the cell yield and viability were recorded. A sample of the cell product was then sent for mycoplasma, gram stain, aerobic/anaerobic, and fungal culture. In addition, the cells were sent to cytopathology in order to confirm the absence of tumor cells.

2.8 Cytokine-Release Assays

Cytokine release by TIL was evaluated at the time of harvest in response either to polyclonal triggering via the CD3-TCR complex or against a selected panel of melanoma tumor cells. Because of a lack of known antigen-specificity in three patients, the potency of these TIL was evaluated only for cytokine secretion in response to stimulation with OKT3 antibody. TIL were added to plates pre-coated with 1 μg/mL of this antibody and twenty-four hours later the supernatants were harvested. IFNγ, TNF-α, IL-2, and IL10 secretion were quantified by ELISA (Pierce/Endogen, Woburn, MA). All assays were controlled using the JKF6 TIL specific for MART-1:27–35 (MART-1).

For those experiments using TIL with known antigen-specificity, a co-culture assay was performed. TIL cells (1×10^5 cells/well), as well as the appropriate effector controls, were plated in a 96-well plate with 1×10^5 stimulator cells. Effector controls included the T-cell clone L2D8 (specific for gp100:209–217 (gp209) as well as the TIL lines JKF6 (specific for MART-1) and AK1700 (specific for autologous tumor). Stimulator cells included HLA-$A2^-$ cell lines (888Mel and 938Mel), HLA-$A2^+$ cell lines (526Mel and 624Mel), and TAP-deficient T2 cells pulsed with MART-1 or gp209. Again, the following day, supernatants were harvested and IFNγ secretion was measured by ELISA.

2.9 Phenotype Analysis

T cells were washed and resuspended at 1×10^7 cells/mL in FACS buffer consisting of PBS +5% fetal calf serum. Staining was done with anti-CD3, anti-CD8, and anti-CD4 antibodies (BD Biosciences). Additional surface markers were evaluated using anti-CD19, CD56, CD27, CD28, CD62L, CCR7, CD25, CD69, CD45RA, and CD45RO (BD Biosciences). For two samples, tetramer binding was assessed by staining with HLA-A2/MART-1:26–35(27L) iTAG tetramer complexes (Beckman Coulter, Immunomics). Cells were washed twice in FACS buffer and analyzed using a FACSCaliber (BD Biosciences) with live/dead gating based on propidium iodide exclusion. Alternatively, some samples were fixed with 0.5 mL of 1% formaldehyde prior to analysis. FACS results were analyzed with FlowJo software (Treestar, Inc., Ashland, Oregon).

3. Results

3.1 TIL with known antigen-specificity can be expanded in a closed system bioreactor

Adoptive cell therapy depends on the large-scale expansion of highly active lymphocyte lines, such as TIL, which has traditionally only been accomplished using T-flasks and gas-permeable bags. To evaluate the potential of the bioreactor, antigen-specific TIL from an HLA-$A2^+$ patient (TIL 2784-1F2 d17) underwent a complete fourteen day rapid expansion from start (inoculation) to finish (harvest). Based on the surface area of the cell bed, a bioreactor was inoculated with a feeders-to-TIL ratio of 200:1 (1×10^9:5×10^6). A separate rapid expansion using the same cell line was started in a flask using a feeders-to-TIL ratio of 100:1 (1×10^8:1×10^6). The yields from the bioreactor and the flask/gas-permeable bag were 3.5×10^9 cells

and 3.0×10^9 cells, respectively. CD4 and CD8 populations were also similar in both harvest populations with approximately 60% CD4 marker expression and 35–40% CD8 marker expression. Additional FACS analysis shown in Figure 2A demonstrates that the MART-tetramer binding by bioreactor-generated CD8+ TIL was similar to CD8+ TIL grown under static culture conditions.

Following the fourteen-day rapid expansion, TIL grown under both conditions were tested in co-culture against HLA-matched (526Mel and 624Mel) and mismatched (888Mel and 938Mel) tumor lines. Figure 2B shows that the bioreactor-generated TIL have similar IFNγ-release and specificity in response to the panel of melanoma tumor cell lines as TIL grown using standard methods. Thus, the results of this experiment demonstrate that TIL with similar surface phenotype and tumor antigen specificity can be expanded using this device when compared directly to control cultures.

3.2 Optimization of rapid expansion in the bioreactor to provide the simplest process for reliable generation of the T-cell product

The closed single-pass perfusion cell production process was evaluated in a series of experiments to derive the simplest method that would reliably produce a clinical quality cell product. To this end we investigated multiple culture parameters including timing of IL-2 introduction, serum concentrations, inoculation densities, and culture duration. Initially we investigated the timing of IL-2 addition to the cultures, because the original REP description delayed the addition of IL-2 until two days after T-cell activation in the presence of anti-CD3 mAb (Riddell and Greenberg, 1990). In bioreactors, IL-2 addition on day 0 or on day2 yielded reliable expansion, and when day 0 was compared directly with day 2 in a single head-to-head experiment, comparable results were obtained (data not shown). Since addition of IL-2 on day 0 represents a logistically simpler approach to cell expansions, this protocol was adopted for subsequent evaluation. It should be noted that the optimal timing and concentration of IL-2 addition was not fully investigated in the bioreactor, and these conditions might be investigated in the future to further improve absolute cell yield.

We next investigated the role of serum concentration on cell yield in the bioreactor. Under conditions of continuous perfusion described above, each bioreactor required seven liters of media over the course of a fourteen-day rapid expansion. From our prior experience with cell production, we have found serum to be both a valuable and expensive resource. Thus, for reasons of economy, it was prudent to attempt to limit serum requirements. Figure 3A demonstrates that cell yields were not substantially different when bioreactors from two different TIL samples were perfused with media containing 1% versus 5% serum. In addition, cell viability was not adversely affected by low serum conditions as each bioreactor product had a viability of greater than 90%.

The role of initial TIL inoculum number was investigated in four experiments using three independent TIL as shown in Figure 3B. In experiments one and two, bioreactors inoculated with 5×10^6 TIL (lower density) were run in parallel with bioreactors inoculated with 1.5×10^7(higher density). In two subsequent experiments (Fig. 3B, Experiments 3 and 4), inoculation densities of 5×10^6 and 2.0×10^7 were compared. In general, there was significant variation in the ability of individual TIL cultures to expand under the conditions of the REP. However, a 3–4 fold increase in the inoculum from 5×10^6 to either 1.5 or 2.0×10^7 TIL per bioreactor on day 0 resulted in a modest 7–40% increase in TIL output at the time of harvest on day 14 ($P<0.05$, Experiments 1–4, Fig. 3-B). These observations indicate an acceptable TIL expansion after 14 days under perfusion culture conditions from a minimum inoculum of 5 million TIL per bioreactor cassette.

Finally, we investigated the length of the rapid expansion in the bioreactor while varying total culture duration from 14 to 20 days as shown in Figure 3C. Three experiments were performed, and in each experiment both a short duration (14–16 days) and an extended duration (18–20 days) in culture yielded a reliable cell product. The extended duration in culture improved the total cell yield per cassette by 32–73 % while providing an additional 1.5 – 2.7 billion TIL per cassette after harvest at the extended duration.

These results led us to establish a standard operating procedure that included the addition of IL-2 on day 0, the use of 1% serum in the bioreactors, inoculation with at least 5×10^6 TIL, and growth for a minimum of 14 days until harvest. These conditions were established to allow the simple, economical, reliable generation of a cell product. Additional investigation of these parameters could be undertaken to optimize yield or other product performance characteristics.

3.3 The closed-system bioreactor led to reproducible and reliable expansion of antigen-specific TIL

Table 1 shows additional rapid expansions that were completed using three different antigen-specific TIL fragments from two different patients. Each bioreactor was inoculated with 5×10^6 cells and in two of the TIL samples conventional control flasks were run in parallel with starting populations of 1×10^6 or 1×10^5 TIL. The average total yield for all bioreactors used in this set of experiments was 3.76×10^9, and a single bioreactor inoculated with TIL 3 resulted in 5.8×10^9 cells which equates to an 1127-fold expansion.

When CD4 and CD8 TIL populations from the two culture conditions were compared by flow cytometric analysis, the final populations from bioreactors and flasks/bags were nearly identical (Table 1). In each case, the final cell product consisted of approximately 90% CD8+ TIL. In addition, the CD8+ cells harvested from the bioreactors had similar MART-tetramer binding to the TIL grown under static culture conditions (Table 1).

Co-culture analysis was performed to evaluate the functional status of the antigen-specific TIL grown in the closed-system bioreactor. Results comparing TIL lines produced in bioreactors compared to control flasks/bags are shown in Table 1. Each bioreactor product demonstrated specific activity against HLA-A2+ matched tumor lines as well as T2 cells pulsed with MART-1 peptide while only minimal background levels of IFNγ were detectable in co-culture against HLA-A2− cell lines and T2 cells without peptide. Also, levels of IFNγ secretion by bioreactor-generated TIL were equal if not higher when compared to controls grown in flasks and gas-permeable bags.

3.4 TIL from the closed-system bioreactor meet all criteria for a current FDA-approved ACT protocol

An alternative to the identification of antigen reactive TIL prior to performing a REP is currently being explored by minimizing the *in vitro* culture period after tumor harvest (Tran et al., 2008). Minimally grown TIL from three patients that were expanded under these exploratory conditions also were investigated using the closed-system bioreactor (Table 2). Each bioreactor was inoculated with 5×10^6 cells and run in parallel with a flask (started at 1×10^6 cells). Total cell yields from the bioreactors ranged from 2.0 to 4.0×10^9 cells. The overall expansion from the bioreactors was 400–800 fold, resulting in total expansions that were 3–7 times lower in bioreactors compared to flask/bag cultures. While some optimization in bioreactor yield was obtained by tuning the growth conditions, the surface area of the bioreactor appeared to be major limitation to yield. The final CD4 and CD8 populations were similar between the two culture conditions.

Since these "young" or minimally expanded TIL were of unknown antigen-specificity, the final product from both bioreactors and flasks/bags was compared by measuring cytokine release in response to OKT3 stimulation. IFNγ secretion was substantial, with levels greater than 10,000 pg/mL in each experimentThe levels of the other type I cytokines, IL-2 and TNFα, were also substantial, while the levels of the type 2 cytokine IL-10 were negligible. Cytokine levels varied slightly within experiments, but fell within the range of experimental variation and there was no obvious superiority of cells from bioreactor-generated TIL comparable to the products from flasks/gas-permeable bags or vice versa. Because these minimally expanded TIL are similar to cell products currently administered in ongoing clinical trials, we evaluated them with a battery of tests as required by the US Food and Drug Administration (FDA) for release of each batch of cells for patient infusion. The viability of the cells harvested from each bioreactor was greater than ninety percent. The cell product from each individual cassette was negative for contamination by microbiological culture and endotoxin assays. Finally, all harvested samples were assessed by a cytopathologist who identified the cell product as tumor infiltrating lymphocytes without detecting the presence of tumor cells.

3.4 Continuous single-pass perfusion allows for stringent control of lactate levels and generates phenotypically similar cells to TIL grown in flasks/gas-permeable bags

Waste media was drawn from the bioreactor's sampling port and from either the flask or gas-permeable bags beginning on day 3 of rapid expansion. Lactate levels were measured by spectrophotometer and perfusion rates were adjusted to keep the lactate level between 0.5–1.0 mg/mL. Previous experiments conducted by Aastrom Biosciences had found expansion and function to be optimal when the lactate level was maintained within this range. Figure 4A shows an average of the lactate levels over a 14-day rapid expansion for bioreactors perfused with 1% and 5% serum. A third trend line shows the average lactate levels from static culture conditions. In all three, lactate rises steadily in the first few days of expansion, but in the case of the bioreactors, adjustments in perfusion rates make it possible to maintain the lactate level within the targeted range while the lactate level in the flasks/bags is consistently higher (>1.0 mg/mL) through the course of rapid expansion. Despite high lactate levels in the flasks, the expansion of TIL in flasks/bags was always similar or superior to the bioreactors.

Using the formula described in the methods section, total lactate was calculated based on the daily lactate level and the daily percent media exchange, via perfusion, in the cell bed compartment. Figure 4B demonstrates the very close relationship between total lactate and eventual cell yield (R^2=0.84). This strong correlation suggests that lactate may be useful not only as a non-invasive indicator of metabolic activity and early culture performance but also as a predictor of eventual cell yield. Examples of total lactate usage from two young TIL cultures that were seeded with five million cells are shown in Figure 4C. The resulting expanded cultures had very similar phenotypic and functional characteristics, but TIL 4 expanded 400 fold during the REP while TIL 5 expanded 840 fold during the REP. Considering that this is a closed-system bioreactor in which cell expansion cannot easily be visualized as in the T-flasks and gas-permeable bags, the additional value of this metabolite as a predictor of cell yield is notable.

The expression of different phenotypic markers by TIL grown in bioreactors was compared to those grown under static culture conditions. This panel of cell surface markers included costimulatory molecules (CD28 and CD27), homing molecules (CCR7 and CD62L), markers of differentiation (CD45RA+ and CD45RO+), CD69 (a marker of activation), and CD25 (the IL-2 receptor α-chain). Figure 5 shows the percentage of CD4 and CD8 lymphocytes from both rapid expansion methods that express these markers. There did not appear to be any significant difference in the phenotypes of these two harvest populations. In general, both CD8 populations resembled the phenotype of the intermediate to late-stage effector cells (i.e. $CCR7^{Lo}$,

CD62L^{Lo}, CD27^{Lo}, CD28^{Lo} CD45RA$^{+/-}$) that we commonly administer to patients as a treatment infusion (Gattinoni et al., 2005;Powell, Jr. et al., 2005). Interestingly, CCR7 expression, which is typically low in a late-stage effector cell population was strongly expressed in a number of samples from both the bioreactors and the flasks/gas-permeable bags.

4. Discussion

TIL-based ACT for patients with melanoma, as well as other T cell based immunotherapies, offers a promising new modality that can mediate curative responses in patients with refractory cancers. These therapies depend on reliable methods to generate safe and effective cell products, but the methods currently available generally require highly trained staff and/or open culture systems, and therefore notably constrain the wider clinical evaluation of these promising therapies. One potential solution to some of these financial, logistical, and regulatory considerations is a closed-system bioreactor capable of the reliable production of a cell product suitable for clinical use. Two bioreactors that have been previously investigated are the hollow-fiber and Wave® systems (Knazek et al., 1990;Tran et al., 2007). One fundamental limitation of both these devices is that the rapid expansion of TIL had to be initiated in flasks or bags before the cells could be transferred to the bioreactor system. This manipulation introduces the chance of contamination and makes both devices less attractive for large-scale clinical implementation.

To address the need for a closed system bioreactor that can accommodate the full REP process, we investigated a single-pass perfusion system. The Aastrom closed-system bioreactor allows for the initiation of the rapid expansion thus eliminating extensive open system manipulation during manufacture of the final product. As far as we know, this is the first example of the initiation and completion of a rapid expansion in a closed-system bioreactor. Although the initiation of the bulk TIL culture from the resected tumor specimen still occurs in an open system, the process of generating tumor-reactive cells can be separated temporally, logistically and conceptually from the REP. After initiation in small batch volumes, the experiments shown here demonstrate that the rapid expansion of TIL with both known and unknown antigen-specificity can be accomplished entirely in a closed-bioreactor system. In addition, these cells either have the same specificity for tumor and peptide targets or have similar cytokine release profiles in response to OKT3 as TIL grown in flasks/gas-permeable bags. Phenotypically, the TIL generated from the bioreactor have the same profiles as those grown under static culture conditions. Finally, all TIL cultures produced in the closed system bioreactors passed stringent Certificates of Analysis, including viability, sterility, identity, and potency assays, identical to those required for cell products for patient infusion in current TIL clinical protocols.

The single-pass perfusion process offers two additional theoretical advantages to static culture for T cell growth, because the media flow and gas exchange are “decoupled” from one other. First, perfusion rates can proceed slowly in order to simultaneously remove metabolites (i.e. lactate and ammonia) while maintaining the “micro-environment” of the cells. Secondly, because gas exchange is independent of media flow, the recirculation of media for the purpose of oxygen delivery (i.e. hollow-fiber systems) and the cell damaging effects of shear forces due to mechanical mixing and sparging (i.e. stirred-culture systems) is either unnecessary or avoided entirely.

One limitation to the Aastrom bioreactor, in comparison to traditional methods of flasks/bags, is the fixed design of the cassette. This inflexibility of design limits the usefulness of the bioreactor in several ways. First, smaller scale versions of the bioreactor are not currently available which complicates optimization and application of the single-pass perfusion method across multiple cellular platforms or clinical protocols due to the expense of side-by-side evaluation at full clinical scale. Additionally, there is limited opportunity for in-process

monitoring of the cell product, which can be a requirement in some cell therapy clinical protocols. In this study we found a direct correlation between lactate production as measured in effluent medium and the yield of viable TIL at the time of harvest based on results from 13 different bioreactor cassettes. Many cell types produce lactate as a result of the shift from oxidative metabolism to the glycolytic pathway during oxygen deprivation. In contrast, several studies indicate that activated T-cells normally derive a very high proportion (>85%) of metabolic energy from the glycolytic pathway via a process termed "aerobic glycolysis" during maximum cell division in the presence of oxygen (Krauss et al., 2001;Brand and Hermfisse, 1997). Our observations are highly consistent with these studies of T-cell metabolism. Thus, total lactate production by rapidly expanding TIL appears to provide an excellent non-invasive and easily measurable metabolic indicator of progressive TIL expansion during the course of culture in the perfusion bioreactor system. These lactate measurements provide a reliable in-process surrogate for lymphocyte growth in the closed system.

The fixed and non-scaleable design of the Aastrom biochamber is a major limitation with respect to the maximum cell yield obtained from a single bioreactor cassette. Due to the fact that the bioreactor is a closed-system, there is no option for the dilutional expansion (e.g. one cassette split to two cassettes) that is used in flasks/bags to maintain a low cell density of $\leq 1–3 \times 10^6$ cells/mL. In contrast to conventional cultures, TIL are expanded in the bioreactor at a progressively increasing cell density until a maximum or "plateau" concentration of 10 – 30 million T-cells per mL is achieved under current operating conditions. Because a bioreactor with a larger surface area is not available, the surface area of the bioreactor was the major limiting factor for cell yield. Other REP components were also evaluated, including larger inoculation densities and longer rapid expansion times. Larger initial cell inocula (1.5 to 2.0×10^7) and longer duration of the REP (18 and 20 days) resulted in higher cell yields. Another potential modification for yield improvement is the periodic redistribution of the cells using the processor during the REP and preliminary experiments suggest that this simple manipulation may also improve bioreactor production. These results are encouraging, and suggest that a combination of higher starting populations, longer times in REP, periodic redistribution and ramped oxygen concentration may yield an optimized final cell number per bioreactor cassette.

The Aastrom closed-system bioreactor is unique in its ability to support previously cultured, antigen specific lymphocytes, including melanoma TIL, over three logs of cell density - from the initiation through the completion of a REP. The expanded TIL are suitable for human infusion in adoptive cell therapy clinical trials by all safety and efficacy assays. Continuous single-pass perfusion utilizing minimal serum requirements provides an environment for non-adherent cell growth, and simple lactate measurements allow for adjustments in media flow that maintain a healthy cellular "micro-environment". Although the cell yields were routinely around 5×10^9 lymphocytes per cassette, this did not justify its use for current Surgery Branch ACT clinical protocols to treat patients with advanced melanoma where target cell numbers for infusion are about 50×10^9. These experiments demonstrate that the single-pass perfusion bioreactor may be aneffective alternative for lymphocyte production for cell therapy applications where smaller cell numbers are targeted.

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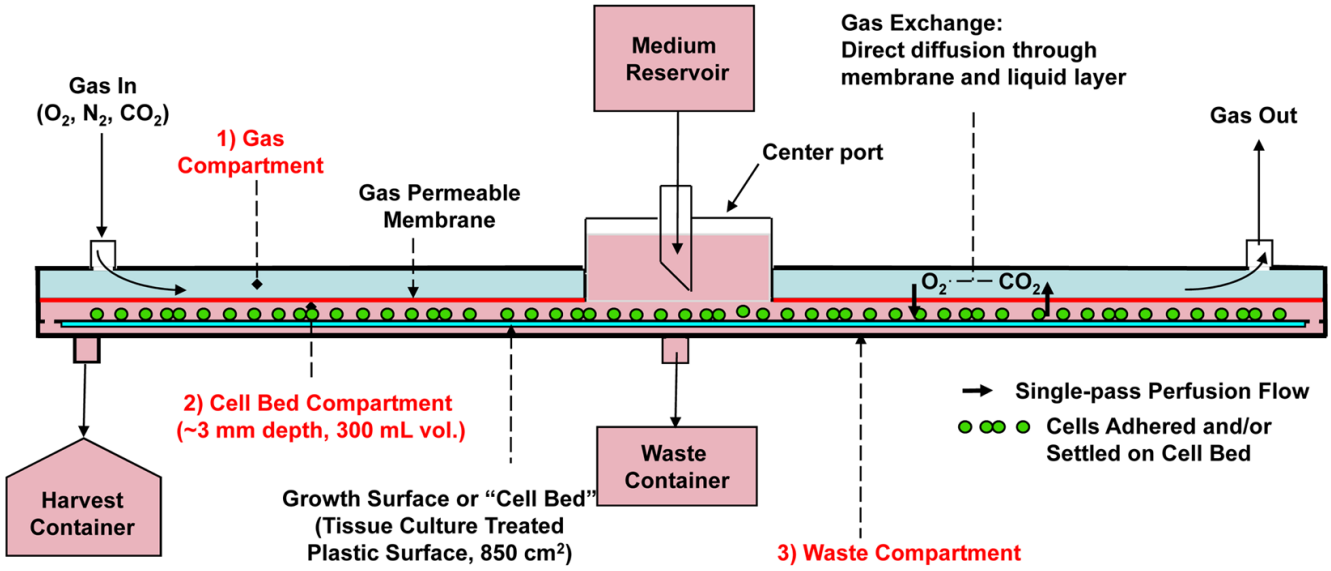

Figure 1.

The closed-system bioreactor is essentially a single, circular chamber. Three compartments of the closed device are shown in cross-section consisting of: 1) gas compartment, 2) cell bed compartment, and 3) waste medium compartment. Single-pass perfusion occurs from the center port of the biochamber and continues radially over the cell bed. Uniform oxygen delivery is achieved independently of the medium exchange rate through a gas-permeable and liquid-impermeable membrane separating the gas and liquid sides of the biochamber.

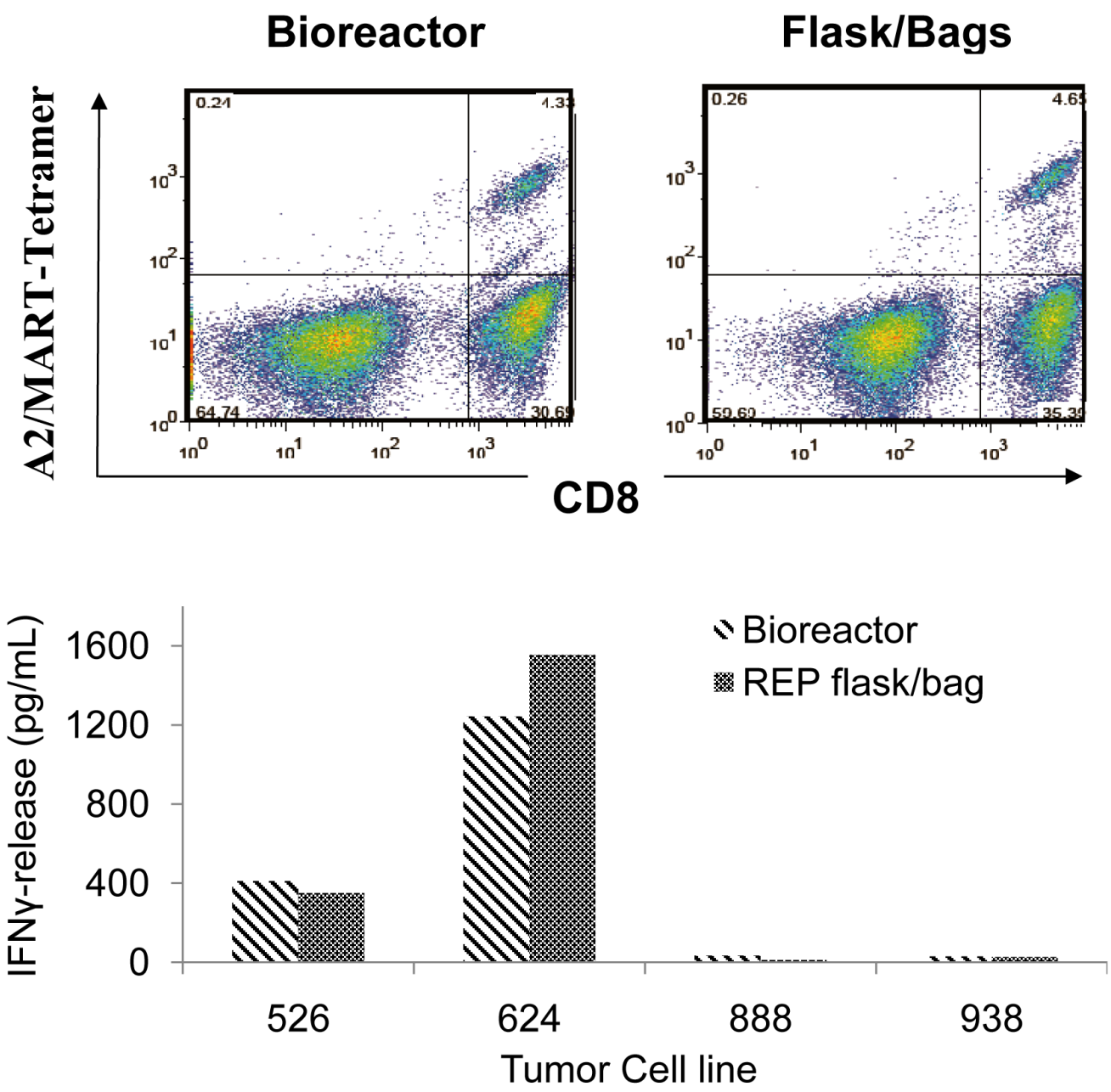

Figure 2.

TIL after expansion in the perfusion bioreactor demonstrate comparable function and phenotype to static T-flasks/bags. A. Flow cytometric analysis of TIL after harvest on day 14 of culture using MART-1 tetramers in combination with anti-CD8 monoclonal antibody. Percentage positive cells are shown in each quadrant. B. Co-culture assay of TIL after harvest from the bioreactor or T-flasks/bags. TIL were evaluated for IFN γ release in a co-culture assay against a panel of MART-1^{+}, HLA-A2^{+} (526, 624) or MART-1^{+}, HLA-A2^{-} (888, 928) melanoma tumor cell lines.

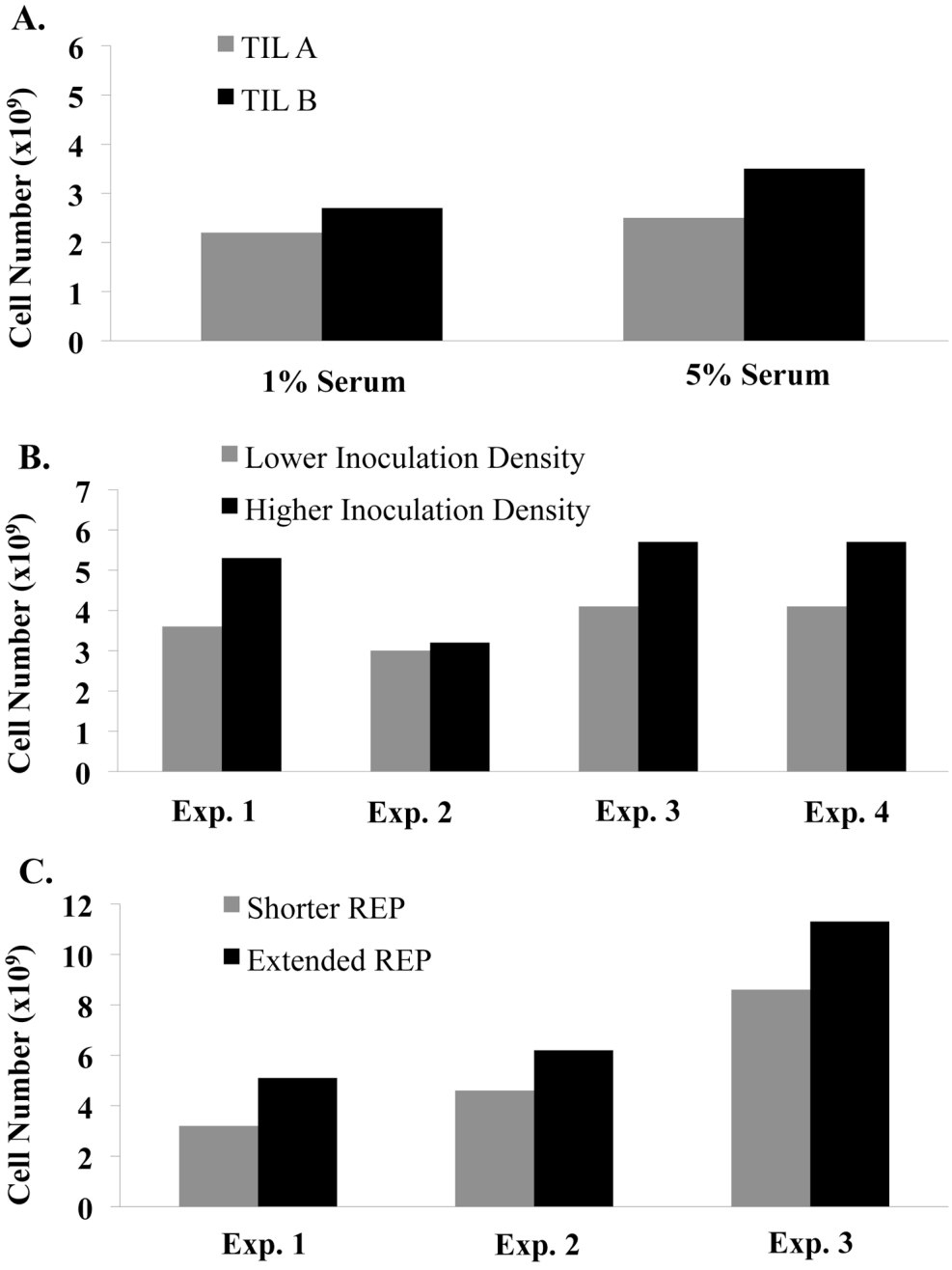

Figure 3.

Optimization of culture parameters to provide a straightforward process for closed-system TIL expansion in the bioreactor. A. *Serum concentration*. Two different TIL isolates designated TIL A and TIL B were expanded in side-by-side experiments by continuous perfusion of medium supplemented with either 1% or 5% human AB serum. B. *Inoculum Density*. TIL were inoculated within each individual experiment at a lower density of 5 million verses a higher density of either 15 million (Experiments 1 and 2) or 20 million (Experiments 3 and 4) TIL per bioreactor cassette. C. *Culture Duration*. TIL were harvested from bioreactors on days 14 vs. 18 (Experiment 1), days 14 vs. 20 (Experiment 2) and days 16 vs. 19 (Experiment 3).

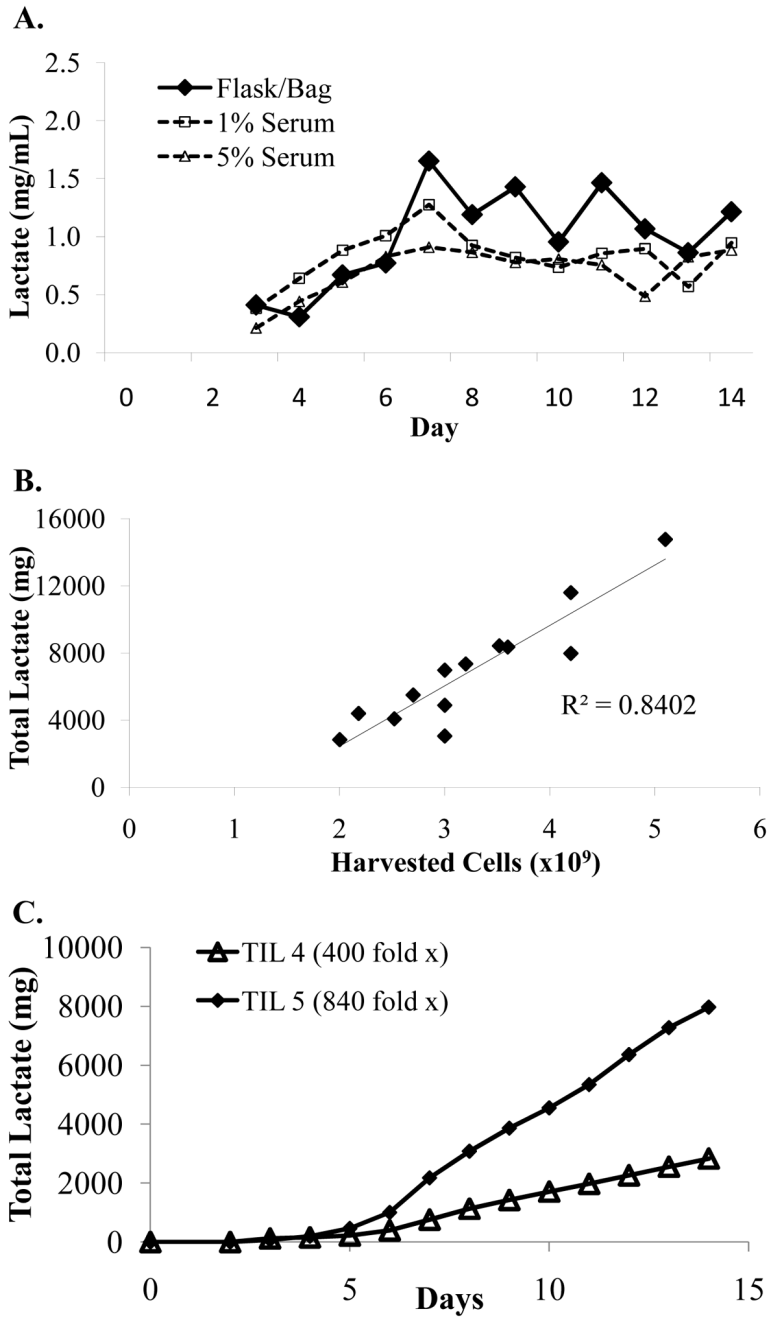

Figure 4.

A. Average lactate levels for TIL grown in 1% and 5% serum was lower over the course of a 14-day expansion as compared to TIL grown in flasks/bags. B. Total lactate produced in an individual cassette correlates strongly with eventual cell yield at harvest. C. Total lactate produced from two similar TIL cultures accumulates steadily over the REP and predicts relative fold expansion.

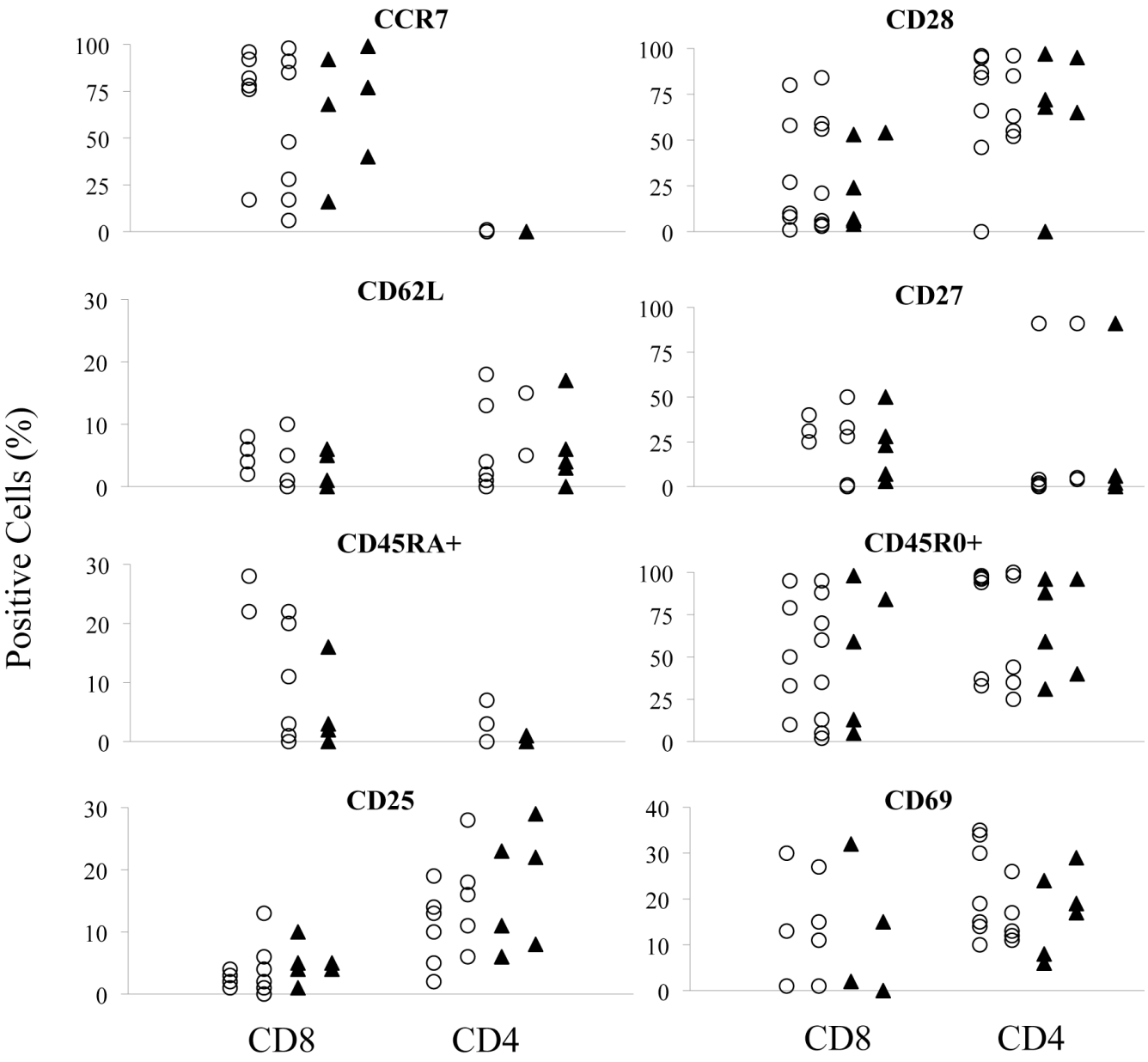

Figure 5.
Phenotypic characteristics of cell products grown in flasks/bags (*triangles*) vs. cassettes (*circles*)were comparable in terms of their expression of a panel of CD markers.. Expression of individual markers (indicated on each plot) varied widely, but no consistent difference between expansion methods was observed for any marker.

Table 1

Rapid expansion of antigen-specific TIL

Effector TIL		Cell No.		Culture Phenotype (% of final cells)[a]			IFNγ Release (pg/mL)					
							HLA A2+		HLA A2−		T2	
		Initial	Final	CD4	CD8	CD8+MART+	526	624	888	938	MART-1[b]	None
TIL 1 (d16)	Cassette 1	5.0E+06	3.6E+09	9	87	39	**32500**	**55600**	500	1664	**51500**	308
TIL 2 (d25)	Cassette 1	5.0E+06	3.0E+09	4	90	5	**4590**	**11450**	34	16	**6790**	208
	Flask/Bag	1.0E+06	9.0E+08	1	90	9	**2930**	**4850**	65	26	>2000	1151
TIL 3 (d20)	Cassette 1	5.0E+06	5.8E+09	11	95	22	**23930**	nd[c]	nd[c]	12	**9042**	26
	Cassette 2	5.0E+06	2.9E+09	10	95	26	**20731**	nd[c]	nd[c]	16	**9402**	25
	Flask/Bag	1.0E+05	6.3E+07	9	96	25	**5993**	nd[c]	nd[c]	13	**2498**	25

[a] Percentage of CD4 and CD8 cells in final population

[b] Not done

[c] MART-1: 27–36

Table 2

Rapid expansion of TIL suitable for patient infusion under existing surgery branch “minimally-cultured TIL” protocol

Effector TIL		Cell Number		Fold Expansion	Culture Phenotype (% of final cells)		OKT3 Stimulated Cytokine Release Assay (pg/mL)			
		Initial ($\times 10^6$)	Final ($\times 10^9$)		CD4	CD8	IFNγ	IL-2	TNFa	IL-10
TIL 4 (d12)	Cassette 1	5.0	2.0	400	8	90	12195	630	397	0
	Flask/bag	1.0	3.0	2800	20	68	12440	1770	17	19
TIL 5 (d13)	Cassette 1	5.0	4.0	840	36	56	13240	550	565	0
	Cassette 2	5.0	3.0	600	35	61	14935	884	>2000	0
	Flask/bag	1.0	2.0	2400	30	66	33545	376	428	0
TIL 6 (d12)	Cassette 1	5.0	3.0	504	20	68	64350	1962	1681	0
	Cassette 2	5.0	2.0	440	38	57	34450	534	922	0
	Flask/bag	1.0	3.0	2600	25	71	84550	2111	692	0