Published in final edited form as:

J Immunother. 2010 October ; 33(8): 759–768. doi:10.1097/CJI.0b013e3181f1d644.

The impact of ex vivo clinical grade activation protocols on human T cell phenotype and function for the generation of genetically modified cells for adoptive cell transfer therapy

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Abstract

Optimized conditions for the ex vivo activation, genetic manipulation, and expansion of human lymphocytes for adoptive cell therapy (ACT) may lead to protocols that maximize their in vivo function. We analyzed the effects of four clinical grade activation and expansion protocols over three weeks on cell proliferative rate, immunophenotype, cell metabolism, and transduction efficiency of human peripheral blood mononuclear cells (PBMCs). Peak lentiviral transduction efficiency was early (days 2 to 4), at a time when cells demonstrated a larger size, maximal uptake of metabolic substrates, and the highest level of proximal TCR signaling engagement. Anti-CD2/3/28 activation beads induced greater proliferation rate and skewed PBMCs early on to a CD4 phenotype when compared to the cells cultured in OKT3. Multicolor surface phenotyping demonstrated that changes in T cell surface markers that define T cell functional phenotypes were dependent on the time spent in culture as opposed to the particular activation protocol. In conclusion, ex vivo activation of human PBMCs for ACT demonstrate defined immunophenotypic and functional signatures over time, with cells early on showing larger sizes, higher transduction efficiency, maximal metabolic activity and ZAP-70 activation.

Keywords

Adoptive Cell Therapy; Melanoma; Ex-vivo activation; Lentiviral Transduction; Immunophenotyping

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Financial Disclosure: All authors have declared there are no financial conflicts of interest in regards to this work.

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Introduction

Adoptive cell transfer (ACT) therapy involves the reinfusion of autologous ex vivo activated and expanded antigen-specific lymphocytes to patients following a host lymphodepleting regimen. Clinical experiences to date with ACT demonstrate significant antitumor activity in patients with metastatic melanoma.^{1, 2, 3} Three main sources of tumor antigen-specific lymphocytes have been investigated in the clinic: i) autologous tumor-infiltrating lymphocytes (TILs) expanded from tumor biopsies,⁴ ii) cellular cloning of antigen-specific lymphocytes expanded from peripheral blood mononuclear cells (PBMCs),^{5, 6} and iii) PBMCs genetically re-directed to become tumor-specific using viral vector-mediated transduction of T cell receptor (TCR) chains.⁷ Among these approaches, TCR engineering provides a promising strategy for the rapid generation of large numbers of tumor-specific lymphocytes in a step-wise and predictable manner.^{7, 8} To generate TCR engineered lymphocytes, PBMCs collected from peripheral blood must be stimulated ex vivo to proliferate and enable retroviral or lentiviral vector transduction with the genes coding for two cancer-specific TCR chains. However, the timing of viral vector transduction and the subsequent cellular manipulation for the selection of 'optimally fit' TCR transgenic T cell preparations remains to be elucidated since a relative paucity of data currently exists on the immunophenotypic and functional signatures of PBMCs during ex vivo activation.

It is likely that the ex vivo expansion and the viral vector transduction to express transgenic TCR genes will alter the phenotype and function of lymphocytes, resulting in changes in their in vivo distribution, persistence, and antitumor activity. Potential scenarios include the inability of T cells to maintain a naïve phenotype with the ex vivo manipulations, the loss of specific memory T cell phenotypes, and the acquisition of late effector or exhausted T cell phenotypes over time. Different T cell subsets have distinct surface phenotypes and functional profiles, and emerging data suggests that they may have markedly different abilities to persist in vivo upon ACT.⁹

Several cell surface markers have been used to define T cell functional phenotypes. Naïve T cells are defined by CD45RA expression and the lymph node homing markers CD62L and CCR7. Evidence supports that the expression of these markers enables naïve T cells to extravasate from high endothelial venules and migrate to T-cell zones of peripheral lymph nodes to be exposed to antigen presented by dendritic cells (DC). In addition, they express CD27 and CD28, two costimulatory receptors that aid in their activation upon TCR recognition of antigen. Antigen-exposed T cells downregulate CD45RA and express CD45RO on their cell surface. T central memory cells (TCM) are antigen-experienced cells (expressing CD45RO), and constitutively express CD62L and CCR7, enabling surveillance for antigen presentation in lymph nodes and preparation for secondary expansion. T effector memory cells (TEM) have downregulated lymph node homing markers, which is thought to enable these cells to populate peripheral tissues and inflammatory sites for immediate response to pathogens. TEM maintain CD27 expression but downregulate surface CD28, subsequently limiting their proliferative capacity upon secondary antigen exposure, but enhancing effector function. This phenotype is even more pronounced in T effector cells (TEFF), which readily release cytotoxic granules and immune effector cytokines upon antigen recognition by their TCR, but have decreased ability to persist long term. TEFF cells are negative for CD27 and CD28, as well as for lymph node homing markers, and express markers of terminal T cell activation such as KLGR-1 and the NK marker CD57.^{9, 10}

The ability to recognize these T cell subsets upon ex vivo expansion for genetic modification should provide important information for the planning of ACT approaches. It is notable that preclinical testing in animal models suggests that ACT of T cells with naïve or

TCM immunophenotypes have shown superior in vivo function. For example, CCR7+CD27+CD28+CD62L+ T cells, a phenotype that is characteristic of TCM cells, demonstrated superior ability to eradicate established tumors in mice when compared to TEFF cells, which produced higher levels of cytokines and cytotoxic activity in vitro prior to ACT.^{9, 11} Similar results were generated in a non-human primate model, where transferred cells with a TCM phenotype were superior to cells with a TEM phenotype in their ability to persist in vivo.¹² Furthermore, T cells with a naïve phenotype obtained from TCR transgenic mice demonstrated enhanced antitumor activity after ACT when compared to their mature T cell counterpart.¹³ In contrast to obtaining lymphocytes from TCR transgenic mice, significant challenges exist in the procurement of large populations of antigen-specific T cells from humans with minimal ex vivo manipulation. Hence, the majority of ACT clinical experiences have used extensively expanded cells ex vivo, generating TEM or TEFF phenotypes for cell transfer.^{8, 14, 15} The selection of these T cells has been frequently based on demonstration of full effector functions ex vivo, including high levels of IFN- γ secretion and cytolytic activity, which are features of TEFF cells. However, this effector phenotype has been a suboptimal predictor of their in vivo performance and is thought to be due the ex vivo expansion process driving cells to an overmanipulated, exhausted phenotype.^{5, 9, 16} The ability to genetically re-direct the specificity of large populations of lymphocytes by TCR gene transfer, with a one-time viral transduction procedure, and the full exploitation of homeostatic proliferation after conditioning regimens to expand cells in the host, provides the option of using minimally manipulated lymphocyte subsets for human ACT protocols.

To better characterize ex vivo expanded lymphocytic populations for ACT, we performed studies on human PBMCs to define the phenotype and function of cells activated under four clinical grade activation protocols. We systematically characterized and evaluated lymphocyte growth and proliferative rate, metabolic and signal transduction signatures, and immunophenotypic markers under these protocols. A protocol using the clinical grade anti-CD3 antibody OKT3 and the gamma chain cytokine IL-2 has been extensively tested in ACT protocols.¹⁷ It is well established that the repeated use of OKT3 in lymphocyte activation and expansion protocols results in vast activation-induced cell death (AICD) in part due to the lack of including co-stimulatory signals. To address this limitation, we also tested anti-CD2/3/28 magnetic beads, which provide CD28 costimulatory molecule engagement. These beads are generated at clinical grade and have been shown to adequately expand T cells for ACT when used with IL-2.¹⁸ We also tested the potential value of adding IL-15 to these activation protocols since ACT in mouse models of CD8⁺ T cells cultured in IL-15 demonstrated greater tumor cytotoxicity than those cultured in IL-2 alone.¹⁹ Our studies demonstrate that ex vivo activated lymphocytes experience an initial phase of metabolic activation followed by marked proliferation. During the metabolic activation phase, these cells can be optimally genetically modified with lentiviral vectors and subsequently expanded ex vivo. The comparison of the four clinical grade protocols tested by us yielded information about slight effects of each one of them on the cell surface phenotype and downstream TCR signaling. However, lymphocyte phenotype was mainly guided by the duration of ex vivo expansion as opposed to a particular activation protocol, and it did not follow the classical definitions of naïve, TCM, TEM and TEFF cells.

Materials and Methods

Cell procurement and activation

PBMCs were collected from a healthy donor under UCLA IRB approval #04-07-063 and cryopreserved as previously described.²⁰ PBMCs were thawed and diluted with RPMI complete media containing 5% human AB serum, and 1% penicillin, streptomycin and amphotericin (Omega Scientific, Tarzana, CA). Cells were washed and subjected to enzymatic treatment with DNase (Sigma, St. Louis, MO) for 1 hour at 37°C, and rested

overnight in a 5% CO₂ incubator. The next day cells were diluted with fresh media to a concentration of 1.5 million cells per ml and activated with 50 ng/ml of anti-CD3 antibody OKT3 (Centocor Ortho Biotech Inc., Horsham, PA) or anti-CD2/3/28 activation beads using a bead-to-cell ratio of 1:2 (Anti-Biotin MACSiBead particles, Miltenyi, Auburn, CA). 300 IU/ml of interleukin 2 (IL-2, gift from Novartis, Emeryville, CA), with or without the addition of 10 ng/ml of IL-15 (gift from Amgen, Thousand Oaks, CA) was added to the groups.

Lentiviral vector production

The 293T cell line was obtained from ATCC (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium with l-glutamine (DMEM, BioWittaker, Walkerville, MD), 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin and streptomycin. The constructs required for the packaging of third-generation self-inactivating lentiviral vectors have been previously described.²¹ The MART-1 TCR chains were a kind gift of Dr. Steven A. Rosenberg (NCI Surgery Branch, Bethesda, MD) and were extracted from a retroviral vector that has been previously described.⁸ Viral titer was determined by assessing viral p24 antigen concentration by ELISA (Coulter Immunotech, Miami, FL) and hereafter expressed as mg of p24 equivalent units per milliliter. One microgram per milliliter of p24 measured in the preparation corresponds to approximately 10⁷ GFP transduction units/ml, as assessed by titration in 293T cells. For each production round, we obtained 10 ml of concentrated virus, at 20-40 mg/ml p24 equivalent, which was cryopreserved at -80°C until ready to use.

Lentiviral vector-mediated gene transfer

Stimulated PBMCs were washed twice with AIM-V (Invitrogen Corporation, Carlsbad, CA) and for each transduction point 1 × 10⁶ cells were mixed with the lentiviral vector supernatant at 1 mg p24 equivalents per milliliter. Protamine sulfate (Sigma), was added at the final concentration of 6 mg/ml, and the transduction plates were incubated overnight at 37°C, 5% CO₂. PBMCs were washed twice with AIM-V medium and seeded at a density of 10⁶ cells/ml in AIM-V, 5% human AB serum and 300 IU/ml of IL-2, with or without the addition of 10 ng/ml of IL-15. Forty-eight hours later cells were harvested for FACS analysis of GFP expression, or MART-1 TCR expression using commercially available MART-1₂₆₋₃₅ MHC tetramers as previously described.²² Transduced PBMCs were re-suspended in 50 µl of adult bovine serum for blocking, followed by staining for the surface membrane antigens CD3, CD8 and CD4 (BD Biosciences, San Jose, CA) for 30 minutes on ice. Flow cytometric analysis was performed in a FACScalibur cytometer equipped with a 488-nm argon laser (Becton Dickinson, Franklin Lakes, NJ).

Flow cytometry staining for surface immunophenotyping and intracellular phosphoprotein staining

Activated PBMCs were resuspended in 100 µl of adult bovine serum (Omega Scientific, Tarzana CA) and stained for 15 minutes at room temperature. A nine-color cell surface staining was used with three cocktail combinations of antibodies in replicate aliquots of the cultures (see Supplemental Table 1). For intracellular phosphoprotein staining, activated PBMCs were fixed with 2% paraformaldehyde (Electron Microscopy Services, Fort Washington, PA), and permeabilized using 90% ice-cold methanol. Fixed and permeabilized PBMCs were washed twice in Dulbecco phosphate buffer saline (DPBS, Mediatech Inc, Manassas, VA), 0.5% bovine serum albumin (BSA, Sigma), and 0.01% sodium azide (Sigma). Replicate aliquots of cultures at different time points were labeled with three cocktails of antibodies in a five-color cell surface and intracellular staining flow cytometry approach (see Supplemental Table 2) for 30 minutes at room temperature in the dark. Antibodies were directly conjugated and used at saturated conditions. For all experiments, a combination of anti-mouse Ig k/Negative Control FBS compensation particles (BD

Biosciences) and PBMCs were used for compensation purposes, and 5×10^5 to 1×10^6 lymphocytes were acquired for each condition. In order to correctly gate the flow cytometry data, the fluorescent minus one (FMO) approach was used.^{20, 23} All samples were acquired on a LSR II (BD Biosciences) and data was analyzed using FlowJo software (TreeStar, Ashland, OR). For extracellular nine-color flow cytometry, Boolean gating was used to calculate the frequency of immunomarkers (Supplemental Figure 1).²⁴ We used Uniform Priority 0, as previously described,²⁵ to depict five independent dimensions of data in a single display for polychromatic plots. The biexponential approach was used in all the plots. For clustering analysis, the flow cytometry data was mean centered, unit normalized, and subjected to unsupervised hierarchical clustering using the average-linkage method based on the Pearson correlation.²⁶ Results were visualized using Java Treeview.²⁷

Metabolic tracer uptake assay

Tritium (^3H)-labeled 2'-deoxy-D-glucose (2'DDG), 3'-deoxy-3'-fluorothymidine (3'FLT) and 2'-deoxy-2'-fluoroarabinofuranosylcytosine (FAC) were purchased from Moravек Biochemicals (Brea, CA). For radioactive tracer uptake assays, we added 0.5 mCi of [^3H]2'DDG, [^3H]3'FLT, or [^3H]FAC to wells containing equal aliquots of 2×10^4 cells from each condition and at each time point in a 96-well tissue culture plate and incubated the plate for 1 hour at 37°C and 5% CO_2 . Glucose free medium was used for experiments with [^3H]2'DDG. Extracellular metabolic tracer was washed off using a multiscreen HTS vacuum manifold system (Millipore, Billerica, MA). 100 μL scintillation fluid (Perkin Elmer, Waltham, MA) was added to each well and tritium counts were measured on a 1450 microbeta trilux microplate (Perkin Elmer). To normalize the amount of radioligand uptake on a per cell basis, 0.05 μCi of probe was placed into 4×10^4 cells/well for 1 hour. After three washes with PBS, the total amount of radioactivity was measured in scintillation counts per minute (cpm). Background counts (0.05 μCi of radioligand placed into an empty well) were subtracted from counts measured in experimental wells. This generated an accurate count of the probe uptake/well. Since each radioligand is characterized by a specific radioactivity in Curies per millimole (Ci/mmol), a simple unit conversion from Ci/mmol to cpm/fmol enabled us to determine the total amount of radioactivity/ 4×10^4 cells.

Statistical analysis

Data were analyzed with GraphPad Prism (version 5) software (GraphPad Software, La Jolla, CA). A Mann–Whitney test or ANOVA with Bonferroni post-test was used.

Results

Clinical-grade lymphocyte activation protocols

Human PBMCs were thawed and activated in triplicate under the following conditions: i) OKT3 antibody plus IL-2; ii) OKT3 antibody plus IL-2 and IL-15; iii) anti-CD2/3/28 activation beads plus IL-2; iv) anti-CD2/3/28 activation beads plus IL-2 and IL-15 (Figure 1a). These four activation protocols provided the platform to analyze cell growth and proliferative rate, metabolic substrate utilization by radioactive tracer uptake, lentiviral transduction efficiency, immunophenotype by polychromatic flow cytometry, and signal transduction with intracellular phospho-protein flow cytometry staining.

Distinct ratios of CD4/CD8 T cell subsets over time with OKT3 and anti-CD2/3/28 beads and CD8 skewing with IL-15

Dynamic changes in the percentage of CD4, CD8, CD4/CD8 double negative, and CD4/CD8 double positive T cell subsets during activation were analyzed. At baseline, thawed PBMCs demonstrated the expected 2:1 ratio of CD4:CD8 T cell subsets, with approximately

65% of CD4+ cells and 25% of CD8+ cells after gating for CD3+ lymphocytes. By day 7, OKT3 and anti-CD2/3/28 beads expanded markedly different T cell subset populations. Under OKT3 influence, 50-60% of total lymphocytes demonstrated a CD8+ T cell phenotype while 75-85% of lymphocytes in anti-CD2/3/28 cultures were CD4+ T cells (Figure 1b). However, the enhanced proliferative rate experienced by lymphocytes stimulated with anti-CD2/3/28 beads yielded a greater absolute number of CD8+ T cells compared to OKT3 cultures at all time points (Figure 2a). Interestingly, we observed reciprocal patterns of differentiation over the 21 days with OKT3 anti-CD3 antibody with IL-2 (CD8+ → CD4+) and anti-CD2/3/28 activation beads with IL-2 (CD4+ → CD8+). The addition of IL-15 directed T cells towards a predominant CD8+ subset in both OKT3 and anti-CD2/3/28 groups. The baseline presence of a small population of CD4/CD8 double negative (but CD3 positive) cells disappeared shortly after placing the cells in culture, with this population reappearing by the end of the culture period. A small population of double positive T cells was also noted by the end of the culture period under all conditions (Figure 1b).

T cell activation with anti-CD2/3/28 beads results in larger cell size and greater proliferative rate compared to OKT3

We analyzed the cell viability and size at different stages of ex vivo activation and expansion using an automated cell counter with trypan blue exclusion. Cell viability was greater than 95% in all groups and at all time points through 21 days (data not shown). Activated cells demonstrated a larger size on days 2 to 4, at a time when cells started proliferation (Figure 2a; $p < 0.0001$; five replicate samples for each time point had very close values, and the error bars are hidden behind the symbols at most time points). Anti-CD2/3/28 beads generated PBMCs that were 1 to 2 μm in diameter larger than those activated with OKT3 ($p < 0.001$). Under all conditions, PBMCs proliferated exponentially from day 2 onwards (note the semilogarithmic plot for cell number in Figure 2a), with anti-CD2/3/28 activation beads showing a greater proliferative rate. This proliferative rate was upheld through day 21. The addition of IL-15 did not generate differences in cell size, but it had an adverse effect in the proliferation of OKT3 cultures ($p < 0.001$ comparing IL-2 alone with IL-2 plus IL-15).

Accumulation of metabolic substrates is positively associated with cell size and negatively associated with proliferative rate

Lymphocyte metabolism is a reflection of cell fitness and function, which we reasoned could be studied by the uptake of metabolic imaging probes.²⁸ We measured the intracellular retention of [^3H]-2'DDG as a measure of glucose metabolism, and [^3H]-3'FLT as reflection of cell proliferation since it is an analogue of thymidine that can be used for PET scanning in humans.²⁹ In addition, we also tested [^3H]-FAC, a recently described PET probe able to detect immune cell activation in mouse models as an additional measure of lymphocyte activation and proliferation.³⁰ In all cases, equal cellular aliquots taken from the cultures were used with results normalized to metabolic tracer uptake on a per cell basis. Interestingly, peak accumulation of all the three metabolic probes was on day 2, with decreased accumulation in cells that were rapidly proliferating thereafter (Figure 2b). Cells activated by OKT3 and anti-CD2/3/28 beads shared similar [^3H]-2'DDG profiles, while activation with the OKT3-containing protocols led to an increased retention of [^3H]-3'FLT and [^3H]-FAC. Addition of IL-15 did not reproducibly affect metabolic substrate accumulation.

Peak lentiviral transduction efficiency correlates with greater cellular size and incorporation of metabolic tracers

In an effort to define optimal scheduling of ex vivo viral vector-mediated genetic engineering of lymphocytes for ACT, we determined the efficiency of lentiviral transduction of CD4⁺ and CD8⁺ isolated lymphocytes under the varying activation protocols. For these studies we used a GFP-expressing lentiviral vector with transduction efficiency analyzed by flow cytometry. Highest transduction efficiency was noted on day 2 (Figure 3a). Interestingly, peak transduction efficiency did not correlate with exponential proliferation, since by day 7 and 14 transduction efficiency levels decreased. The addition of IL-15 did not influence transduction efficiency. Having defined the best timing for lentiviral transduction using a marker gene, we next tested the effects of these different protocols on the transduction efficiency and surface expression of a MART-1 melanoma antigen HLA-A*0201-restricted TCR induced by a lentiviral vector constructed to express the alpha and beta chains of this TCR. T lymphocytes activated with anti-CD2/CD3/CD28 beads had higher surface expression of the TCR in comparison to cells activated with OKT3 (Figure 3b). Interestingly, transduction efficiency was higher in CD4⁺ T cells in comparison to CD8⁺ T cells, which is a reproducible observation in PBMC from other donors (data not shown).

Analysis of signaling networks downstream of surface receptors during ex vivo expansion

Phosphorylation of the protein tyrosine kinases zeta-chain-associated protein of 70 kDa (ZAP-70) and leukocyte-specific protein tyrosine kinase (Lck) are reflective of signal transduction upon surface TCR activation.^{31, 32} Maximal phosphorylation of ZAP-70 in lymphocytes was observed on day 2 post activation in all groups (Figure 4a). There was a six-to-eight fold peak increase in phosphorylation in ZAP-70 in CD4⁺ T cells and a ten-to-fifteen fold peak increase in phosphorylation in ZAP-70 in CD8⁺ T cells, irrespective of the culture system. The pattern of Lck phosphorylation was markedly different, with day 7 demonstrating peak phosphorylation levels and a decrease almost back to baseline by day 15. TCR signaling downstream of these two src kinases is followed by the activation of the mitogen-activated protein kinase (MAPK) and the PI3k/Akt signaling pathways (Supplemental Figure 2). We found evidence of higher levels of phosphorylation of p38 and Erk upon T cell expansion when compared to Akt. We also analyzed the phosphorylation status of signal transducers and activators of transcription (STAT), which are important signaling molecules downstream of cytokine receptors and also of the MAPK pathway. There was a late (day 7) increase in pSTAT6 in proliferating lymphocytes regardless of the activation protocol, while the rest of pSTATs were relatively constant throughout the ex vivo expansion cultures. Similar results were observed at all culture conditions.

Multicolor surface phenotyping of ex vivo expanded lymphocytes

We next asked whether lymphocyte surface markers for activation, lymph node homing, and memory phenotypes that segregate T cell functional subsets were differentially expressed with the four activation protocols. PBMCs obtained at baseline and at 4 time points during ex vivo activation were evaluated by multicolor surface phenotyping using 3 different combination groups of eight surface markers. This strategy enabled the simultaneous analysis of 5 surface markers at each time point on CD3⁺ lymphocytes gated based on CD4 or CD8 positivity. To interrogate whether lymphocyte phenotype subsets segregated by activation protocol or by culture day, we subjected the flow cytometry data to unsupervised hierarchical clustering. As depicted by heatmaps, the major driver for clustering surface phenotypes for both CD4⁺ and CD8⁺ cells was time spent in culture, rather than the activation protocol (Figures 5 a, b). For CD4⁺ T cells, a phenotype of CD25⁺CD27⁺CD28⁺CD44⁺ and CCR7[−]CCR5[−] predominated on culture day 2, while cells with a phenotype of CD45RO⁺HLA-DR⁺ and CD127[−]CD137[−] were predominantly

observed on culture day 21. The surface phenotype was different for CD8 cells, with a phenotype of CD27+CD28+CD137+ and CCR7– predominating on day 2, and CCR7+HLA–DR+ and CD137– by day 21. These phenotypic changes were common with the four activation protocols. However, cells with a CD4+CD25+CD127– phenotype, which has been used to phenotype T-regulatory cells (Tregs), were elevated on day 15 with the OKT3 plus IL-2 protocol (Supplemental Figure 3).

Analysis of CD8+ CD27+ CD28+ T cells as a function of time

We finally asked how our findings compared with a current hypothesis that lymphocytes co-expressing CD27 and CD28 are better suited for ACT since they may impart early effector function.^{11, 14} We analyzed CD27 and CD28, as well as the lymphoid homing marker CD62L, and two markers of terminal effector differentiation, CD57 and CD95, in gated CD8+ T cells (Figure 5c). Baseline cultures prior to activation consisted of a main population of cells with a TCM phenotype (CD27+, CD28+, CD62L+), but with the concomitant expression of Fas receptor (CD95+). By day 2 there was an emergent population of cells with a CD27+CD28–CD95+ and decreased CD62L phenotype, consistent with TEM cells. By day 7, we observed a further significant shift towards this population of cells but with higher CD62L expression. By day 15, the population subsequently shifted to CD27–CD95+, which are markers consistent with a T effector phenotype. Paradoxically, by day 21 there was re-expression of CD62L in cells that were mostly CD27– CD28+ CD95+.

Discussion

The ability to obtain high transduction efficiencies in large batches of activated PBMCs for human cell therapy⁸ makes it possible to limit the ex vivo expansion period while achieving numbers of tumor antigen-specific lymphocytes that are in proportional keeping with the cell transfer load required to induce tumor responses in mice.¹⁷ The limitation of ex vivo manipulation is important since earlier lymphocytic populations with shorter ex vivo expansions are likely to optimize in vivo function. Hence, this work was aimed at defining key features of activated lymphocytes that may enable the identification of optimized conditions for the generation of clinical grade T cell therapies. Our major findings were that ex vivo activation of human PBMCs demonstrated defined and predictable phases under all tested protocols, and that lymphocytes early on in culture demonstrated larger sizes, greater metabolic activity, higher lentiviral transduction efficiency, and unique immunophenotypic signatures that may be more advantageous for recipients of ACT therapy. The generability of the findings from this study is limited by the fact that cells from only one healthy donor were used throughout this work. Even though all studies were internally controlled and included multiple replicates and overlapping readouts, it is possible that a certain activation protocol may be more effective in PBMC from some subjects and not others.

Lymphocyte size is an essential attribute that reflects cellular fitness and function.^{33, 34} Cell size, as demonstrated with larger cell diameter noted early on in the ex vivo activation process, and cell cycle, as shown with the rapid proliferation starting two days after the ex vivo activation, are coordinated but distinct processes.³⁵ During the early activation phase, anti-CD2/3/28 activation beads generated cells that acquired a larger cell size phenotype and subsequently demonstrated enhanced proliferative rate. However, this did not translate into major differences in metabolic tracer uptake or in their phenotype. The observation of increased retention of [³H]-3'FLT and [³H]-FAC by lymphocytes activated with OKT3-containing regimens could be explained by a relative decrease in mass per given cell in the anti-CD2/CD3/CD28 bead group, and hence less retention of radioactive tracer since they experienced greater proliferative rate. However, the findings with early FLT uptake and decreased during exponential growth are unexpected. FLT uptake most accurately reflects

human thymidine kinase-1 (hTK1) activity, an enzyme expressed during the DNA synthesis phase of the cell cycle. TK1 activity has been reported to be highest in proliferating cells and low in quiescent cells.³⁶ However, our data suggest it may be highest in the initial culture period prior to and in preparation for (building up stores) lymphocyte proliferation. The same pattern was noted with FAC uptake, which reflects deoxycytidine kinase (DCK) activity, the enzyme responsible for the initial phosphorylation and intracellular trapping of this PET tracer.³⁰ An additional explanation for the higher accumulation of FLT and FDG before exponential proliferation is that the surface expression of transporters for these nucleoside analogues may be higher during the initial culture period; this possibility was not explored in this work. It is of particular interest that peak lentiviral vector-mediated transduction efficiency was noted during the early stage of cell proliferation concordant with the higher metabolic tracer uptake.

CD4/CD8 skewing seen with varying activation protocols and on different days may result in lymphocytes with distinct phenotype and function for ACT. Preparations with a higher percentage of CD4+ T cells may be better equipped for supporting CD8+ T cells in vivo.³⁷ In this regard, the anti-CD2/3/28 bead protocol without the addition of IL-15 generated higher absolute numbers and proportions of CD4+ T cells during the first two weeks of culture. Therefore, it would be interesting to test this activation protocol in ACT experiments. We expected anti-CD2/3/28 activation beads to costimulate as well as provide TCR signaling by non-specific CD3 engagement, resulting in markedly different lymphocyte phenotypes when compared to activation with OKT3 (lacking the CD28 costimulation). However, our findings suggested that the days spent in culture had a greater influence on immunophenotype than the specific T cell activation and expansion conditions. Of note, the high concentrations of IL-2 used in all the tested conditions may have an overriding effect to potential benefits that may be derived from adding IL-15. It is certainly possible that lower concentrations of IL-2 would have allowed noticing beneficial effects of adding IL-15, which was not evident in our studies.

The day 2 immunophenotype was markedly different compared to day 0, suggesting a rapid change in cell surface marker expression upon ex vivo expansion. It is interesting to note that PD-1, a marker associated with T cells that have progressed beyond their effector phenotype and display evidence of exhaustion³⁸ did not consistently increase with the progression of the cultures until day 21. Both CD4+ and CD8+ T cells almost uniformly lacked the lymph node homing marker CCR7 by culture day 2, but this marker increased on the surface of the CD8 subset gradually and peaked at 3 weeks. Although there are several models to explain the progression between effector and memory cell phenotypes,³⁹ this observation with ex vivo activated and expanded cultures would suggest that peripherally-circulating TEFF cells can acquire a phenotype more consistent with centrally circulating lymphocytes with long term cultures.

There is limited published data to date on the phenotype of TCR transgenic lymphocytes administered to patients. The most complete is from Johnson *et al.*,⁸ where cells used for ACT had low CD27, intermediate CD28, low CD45RA and high CD45RO. Our analysis did not readily detect a significant population of cells with this phenotype (see Figure 5c). However, their approach included the addition of a rapid expansion protocol (REP) involving expanded cultures in IL-2 and allogeneic feeder cells after the initial culture in OKT3 and IL-2. The REP protocol³⁸ results in further expansion of cells by one to two logs, which may generate functional phenotypes different from those described herein. Interestingly, in the Johnson *et al.* work, the ratio of CD45 isoforms changed in TCR transgenic cells recovered from the peripheral blood of patients, with the majority reverting to a CD45RA+ phenotype. This suggests that the functional phenotype of cells prior to ACT

changes with cell expansion in a lymphodepleted host, as previously described in experiences analyzing the process of homeostatic proliferation in murine models.³⁹

Finally, the advent of multicolor flow cytometry poses challenges in conceptualizing and depicting the concomitant analysis of multiple markers simultaneously expressed by single cells analyzed by flow cytometry. If depicted and followed individually or in pairs, the added value of detecting multicolor staining in single cells is lost. With our data, we found that heat map representations of unsupervised hierarchical clustering revealed how cell surface phenotypes segregated based on culture conditions and time. Using this approach, we concluded that the time spent in culture has a greater impact on cell phenotype than the four activation and expansion protocols tested here. This surface phenotype data may aid in the future in interpreting the functional phenotype of adoptively transferred cells to patients.

This work was undertaken in preparation for a clinical program using TCR transgenic lymphocytes for ACT therapy in patients with melanoma (clinical trial registration number NCT00910650). Based on the data presented herein we made decisions for the ex vivo expansion and viral vector transduction of PBMC, with the caveat that data from a single healthy donor may not fully recapitulate what can be encountered when activating and gene modifying PBMC from a series of patients with advanced melanoma. Viral vector transduction to insert TCR genes into lymphocytes is performed on days 2 and 3 after initiating ex vivo PBMC expansion to optimize transduction efficiency. The observation that the time in culture was the major determinant of T cell functional phenotype, as opposed to a particular ex vivo expansion protocol, led us to decide to use OKT3 and IL-2 as the activation protocol given its prior successful use in clinical trials of TCR engineering ACT. Since the production of clinical-grade OKT3 has been recently discontinued, we are using OKT3 from a commercial vendor with additional lot release testing for purity and potency as defined in the investigator new drug (IND) application filed with the FDA. The marked changes with expanded culture in vitro and the emergence of cellular populations with non-physiological phenotypes led us to select a limited (7-day) expansion ex vivo with the hopes to optimize in vivo proliferation after ACT to lymphopenic hosts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Disclosure of Funding Support: This work was supported by grants from the National Institutes of Health awards P50 CA086306 and U54 CA119347, and the California Institute for Regenerative Medicine (CIRM) New Faculty Award RN2-00902-1 and The Fred L. Hartley Family Foundation (to A.R.). P.C.T. was supported by the UCLA Scholars in Oncologic Molecular Imaging (SOMI) program. R.C.K. was supported by the V Foundation-Gil Nickel Family Endowed Fellowship in Melanoma Research. N.A.G. is a postdoctoral trainee supported by the UCLA Tumor Biology Program T32 CA009056. T.G.G. is an Alfred P. Sloan Research Fellow. The UCLA Flow Cytometry Core Facility is supported by the NIH awards CA-16042 and AI-28697.

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Figure 1. Clinical-grade lymphocyte activation protocols and their effects on CD4/CD8 T cell subsets as a function of time

(A) Schematic of the four clinical grade compatible activation protocols. (B) CD4 positive, CD8 positive, CD4/CD8 double negative and CD4/CD8 double positive T cell subsets (all gated on CD3+ cells) analyzed over time with the four activation protocols. Respective percentages of varying T cell subsets are represented over 21 days of activation as analyzed by flow cytometry.

Figure 2. The effects of ex vivo activation protocols on lymphocyte size, proliferative rate, and metabolism

(A) Cell diameter and proliferation under the four varying protocols. Cell size and counts were obtained using an automated cell counter with trypan blue exclusion. (B) Metabolic probe accumulation by ex vivo activated lymphocytes. Equal aliquots of 2×10^4 cells from the different cultures at different time points were incubated with [³H]-labeled metabolic probes for one hour, after which the trapped intracellular radioactivity was measured. Error bars represent means $\pm$ s.e.m. of three independent experiments.

Figure 3. Lentiviral transduction efficiencies in human lymphocytes as a function of time and culture system

(A) The percentage of GFP expressing CD4 and CD8 lymphocytes over time is depicted. Transduction efficiency was determined using flow cytometry after transducing cells with a lentiviral vector expressing GFP. (B) MART-1₂₆₋₃₅ tetramer assay to assess surface TCR expression of CD3+ T cells activated with OKT3 or anti-CD2/3/28 and transduced with a lentiviral-MART-1 TCR vector.

Figure 4. Phosphorylation of src protein tyrosine kinases (ZAP-70 and Lck) in activated human lymphocytes over time in the presence of IL-2 only

Fold change in phosphoprotein levels for p-ZAP-70 (A) and p-Lck (B) in CD4 and CD8 T cells as a function of time under OKT3 and anti-CD2/CD3/CD28 is depicted.

Figure 5. Multicolor surface immunophenotyping of ex vivo activated human lymphocytes
 Unsupervised clustering of heat maps constructed with data from multicolor surface phenotyping of CD4 (A) and CD8 (B) cells using 3 different combination groups of surface markers (see Supplemental Tables 1 and 2). Each column represents cells activated with the indicated stimuli for the indicated time and rows represent the measured surface phenotypes. Red and green indicate high and low percentages, respectively, of cells described by the indicated surface phenotype. Each column sums to $3 \times 100\%$, for the 3 combinations of surface markers. The predominant CD4 phenotypes of CD25+CD27+CD28+CD44+CCR7-CCR5- on day 2 and CD45RO+CD127-CD137-HLA-DR+ on day 21, and CD8 phenotypes of CD27+CD28+CD137+CCR7- on day 2 and CD137-CCR7+HLA-DR+ on day 21 are indicated by red boxes. (C) Time-course analysis of gated CD8 cells in the presence of IL-2 only. Expression of CD27 (y axis), CD28 (x axis), CD57 (green), CD62L (red) and CD95 (blue) in a five dimensional polychromatic plot is depicted.