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CD34-Based Enrichment of Genetically-Engineered Human T Cells for Clinical Use Results in Dramatically Enhanced Tumor Targeting

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Abstract

Objective clinical responses can be achieved in melanoma patients by infusion of T cell receptor (TCR) gene transduced T cells (1, 2). Although encouraging, the therapy is still largely ineffective as most patients were unable to benefit from treatment. That only a minority of the infused T cells were genetically modified and that these were extensively expanded *ex vivo* may have prevented their efficacy (3). Here, we developed a novel selection technology that allows rapid and efficient enrichment of genetically engineered cells for clinical use. Both CD8⁺ and CD4⁺ T cells transduced with retrovirus encoding two TCR chains and a truncated CD34 (CD34t) molecule efficiently co-expressed all the transgenes on the cell surface. Importantly, over 5-fold enrichment of both the frequency of transduced cells and the specific anti-tumor reactivity of the effector population could be achieved by the magnetic beads-based enrichment procedures readily available for hematopoietic stem cell isolation. This CD34t-based technology will improve the feasibility of adoptive transfer of clinically relevant effectors. The effectiveness of the enriched redirected T cells may further be superior due both to their higher reactivity and the reduced *ex vivo* culture period necessary, resulting in less exhausted cells better capable of persisting *in vivo*.

Keywords

TCR; Transduction; Selection; Isolation; Immunotherapy; Melanoma

Introduction

A high proportion of melanoma patients given non-myeloablative chemotherapy prior to the transfer of autologous tumor infiltrating lymphocytes (TIL) experience objective clinical responses (4, 5). While providing an important proof of concept, this treatment regimen has yet to move beyond the experimental phase at the NCI because it remains difficult to isolate and expand tumor reactive autologous T cells from most cancer patients. Moreover, even

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when possible, the process of isolating and expanding tumor reactive T cells from cancer patients to therapeutic numbers is complicated, time consuming, and regrettably many patients progress during this time becoming too sick to treat. Notably, extended *ex vivo* culture of T cells, which is required due to the low original numbers of precursors of most tumor reactive TIL cultures, may in fact be detrimental to their survival *in vivo* and thus their therapeutic efficacy (3). Many of the factors that limit the use of adoptive TIL transfer may be circumvented by instead using genetically modified T cells.

We, and others, have demonstrated that normal PBL-derived T cells can be engineered to express T cell receptor genes isolated from reactive T cell clones, causing their reactivity to be redirected, using retroviral vectors (6–9). By transducing normal patient derived peripheral blood T cells with TCR genes, autologous effector cells can be generated much faster and from any type of patient, regardless of their own capacity to spontaneously mount immune responses. The first reports using TCR gene modified T cells in patients were published in 2006 (1, 2). The most important conclusion from these trials was that treatment was both feasible and safe. Notably, a few objective clinical responses were also observed. A recent study evaluated effectors transduced with TCRs with much higher affinity, notably this led to increased frequency of objective clinical responses (10). However, the clinical results with the high affinity TCRs were less promising than those obtained with TIL suggesting that TCR transduced PBL-derived T cells are less effective than TIL.

Although these early results are encouraging, TCR gene modified T cell therapy is still largely ineffective. Several factors may prevent TCR-gene modified T cells from being efficacious. In the absence of selection, only a minor proportion of the infused T cells may be genetically modified (2), and most cells distributed thus lack antigen reactivity. Importantly, pre-clinical *in vivo* studies further indicate that the contaminating non-transduced cells may actively impair the clinical efficacy of the redirected cells (11, 12). Also, to reach therapeutic numbers, it was necessary to culture all T cells extensively *ex vivo* prior to infusion. Prolonged *in vitro* culture may however be detrimental to the therapeutic efficacy of the T cells (3, 13). A strategy that implements a rapid and efficient enrichment of clinical grade genetically modified T cells from the bulk T cell population, would circumvent these obstacles and improve the efficacy of immunotherapy using TCR-gene transduced cells. In a lentiviral delivery system this selection technology will likely also allow rapid engineering and selection of naïve resting T cells, which may revolutionize adoptive T cell therapy. Current methodology requires antibiotic-based selection (neomycin phosphotransferase/neomycin analogue G418) for transduced cells. While G418 selection will increase the percentage of TCR transduced cells administered to the patient, it will also increase the culture duration (14, 15) and may thus impair the therapeutic efficacy of the T cells (3, 13). Also, the bacterial neomycin phosphotransferase enzyme that mediate resistance to G418 (*neo^r*) is often immunogenic *in vivo* (16). Alternative markers for gene modified cells, such as enhanced green and yellow fluorescent proteins may also be targets for specific cytotoxic T cell responses *in vivo* (17). This severely limited the possibility to get long term persistence of the effector cells, which was described to correlate with clinical effectiveness (18). Therefore, novel means of gene marking and enrichment for genetically altered cells are needed to provide patients with large numbers of non-immunogenic and non-exhausted TCR gene modified T cells.

In the current study we engineered a novel selection cassette to apply an improved type of enrichment technology for redirected cells. T cells transduced with a truncated version of the human CD34 molecule (CD34t) successfully co-expressed it with the introduced TCR. TCR transduced T cells could be readily enriched 5 to 7-fold based on their surface expression of CD34. The CD34 selected T cells had a corresponding 5 to 6-fold increase in specific reactivity against tumor cells. Notably, all of this can be achieved using existing technology

designed to isolate clinical grade CD34⁺ hematopoietic stem cells for allogeneic stem cell transplantation, which is readily available in the clinic. This novel enrichment technology has the potential to improve both the feasibility the effectiveness of adoptively transferred TCR-gene modified T cells. Furthermore, this technology can be used to enrich and improve the effectiveness of almost any genetically modified cell.

Materials and Methods

Generation of single transcript 2A-linked TCR $\alpha\beta$ encoding vectors

We incorporated a “self-cleaving” 2A peptide, derived from porcine teschovirus-1 (P2A) (19), supplemented with a GSG amino acid linker, just downstream of the 3′ end of the TCR α -chains by polymerase chain reaction (PCR) amplification using TCR specific V α 5′ primers containing a *SaI* site and a common 3′ primer encoding the entire P2A molecule (GGGCCCAGGACCGGGGTTCTCCTCCACGTCTCCTGCTTGCTTTAACAGAGAGAA GTTCGTGGCTCCGGATCCACCGGTGCTGGACCACAGCCGAG). Similarly, a 30–36 bp complementary sequence was introduced upstream of the 5′ end of the TCR β -chain of the respective TCR using C β 1 or C β 2 specific 3′ primers containing a *XhoI* site and TCR specific V β 5′ primers (GACGTGGAGGAGAACCCCGGTCTCTGGGCCCCGGCACAAGGTTGTTCTTCTATGT G for the TIL 1383I TCR and GCAGGAGACGTGGAGGAGAACCCCGGTCTCTGGGCCCCACTATCAGGCTCCTCTGC TACATG for the HCV cl 3 TCR). The resulting products were used as templates in another PCR, using the same V α 5′ primers and the C β 1 or C β 2 primers respectively, creating two P2A-linked full-length TCR α P2A β fusion cDNA molecules. These were ligated into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into *Escherichia coli* TOP 10 competent bacteria (Invitrogen). Ampicillin resistant bacterial clones were screened for presence of the cDNAs by PCR using the respective cloning primers and recombinant clones re-inoculated. Plasmids were extracted by commercial miniprep kits (Qiagen, Valencia, CA) and the sequence of all the constructs was determined using BigDye terminator cycle sequencing kits (Applied Biosystems, Foster City, CA) and analyzed using an ABI Prism310 Genetic Analyzer (Applied Biosystems). All mutations present in the cDNA that would cause changes in the amino acid sequence, were corrected by a site-directed mutagenesis kits (Stratagene, Cedar Creek, TX). Similarly, undesired internal restriction sites were removed. The corrected constructs were denoted 1383I α P2A β and HCVcl3 α P2A β , respectively. The SAMEN CMV/SR α retroviral vector backbone was designed specifically to co-express two separate genes (e.g. the TCR α and β chains) in T cells (20). For the current studies this vector was modified using the unique *SaI*, *XhoI* and *BamHI* restriction sites present in this vector. The internal SR α -promoter was removed by insertion of the *SaI* and *XhoI* excised 1383I α P2A β or HCVcl3 α P2A β cDNA between the *SaI* and *XhoI* sites of the vector. Thus, a single transcript was driven by the hybrid 5′ long terminal repeat (LTR), consisting of the human CMV enhancer and promoter fused to the Moloney murine leukemia virus (MMLV) 5′ LTR in the retroviral vectors denoted SAMEN-1383I α P2A β and SAMEN-HCVcl3 α P2A β (Figure 1A).

Cloning of CD34t and generation of CD34t-linked TCR $\alpha\beta$ encoding retroviral vectors

The CD34 molecule contains a signaling domain. To prevent the selection marker from influencing the function of the transduced cells a truncated version of the CD34 (CD34t) molecule was used. It consists of the extracellular and transmembrane regions of the protein and was cloned via reverse-transcriptase PCR from human cDNA (ADDITIONAL INFO FROM JEFF?). PCR products were then cloned as described above. Furthermore, the 5′ end of the CD34t was complemented with both the “self-cleaving” 2A sequence of *Thosea asigna* virus (T2A), shown to allow expression of several proteins from a single polypeptide

(19), supplemented with a GSG amino acid linker and the 3' end of the respective TCR's β -chain using PCR. Two partially overlapping 5' primers was used for each of the two TCRs, (GCTGGTCAGCGCCCTTGTGTTGATGGCAATGGTCAAGAGAAAGGATTTTCAATT CGGCTCAGGCGAGGGCAGAGGCAGTCTGCTAACATGCGGTGATGTCAAGAAA ATCCTGGCCCCACCGCGGGGCTGGACCGCGC and GCCGAGGCCTGGGGTAGAGCAGACTGTGGCTTTACCTCGGTGTCCTACCAGCAA GGGGTCCTGTCTGCCACCATCCTCTATGAGATCCTGCTAGGGAAGGCCACCCTG TATGCTGTGCTGGTCAGCGCCCTTGTGTTG) compatible with the constant region (C β 1) of the TIL 1383I TCR β -chain and (GGTCAGTGCCCTCGTGCTGATGGCCATGGTCAAGAGAAAGGATTCCAGAGGCG AATTCGGCTCAGGCGAGGGCAGAGGCAGTCTGCTAACATGCGGTGATGTCAAG AAAATCCTGGCCCCACCGCGGGGCTGGACCGCGC and GCCGAGGCCTGGGGTAGAGCAGACTGTGGCTTCACCTCCGAGTCTTACCAGCAA GGGGTCCTGTCTGCCACCATCCTCTATGAGATCTTGCTAGGGAAGGCCACCCTGT ATGCCGTGCTGGTCAGTGCCCTCGTGCTGATG) containing part of the constant region (C β 2) of the HCV cl 3 TCR β -chain. The common 3' primer contained a *Bam*HI site (AAATCTAGAGTCGACGCGGCCGCGGATCCTCATGGTTCTAGTTCCAGCCTTTC). Again, the PCR products were cloned as described above and the constructs were sequenced using an ABI Prism310 Genetic Analyzer (Applied Biosystems). All undesired restriction sites, including an internal *Bam*HI sites, as well as mutations present in the cDNA were removed by site-directed mutagenesis (Stratagene). To allow selection of the TCR transduced cells based on their expression of the CD34t molecule, we then introduced this into the SAMEN-1383I α P2A β and SAMEN-HCVcl3 α P2A β vectors (Figure 1B). Using a conserved *Stu*I site in the constant region of the β -chains of human TCRs and the *Bam*HI site at the 3' end of the CD34t-inserts and the vectors we replaced the internal ribosomal entry site (IRES)/neo^r segments while creating the final retroviral vectors designated TIL 1383I TCR/CD34t and HCV cl 3 TCR/CD34t, that expressed a single transcript encoding one polypeptide that cleaves itself into three functional proteins.

Cells

Leukopheresis from healthy donors, obtained following informed consent, were purchased from Research Blood Components, LLC (Brighton, MA). Peripheral blood mononuclear cells (PBMC) were isolated using lymphocyte separation medium (Mediatech Inc., Herndon, VA) density gradient centrifugation and frozen at 100×10^6 cells/ml. Upon thawing, PBL was isolated after a two hour plastic adherence depletion of PBMC. PG13 retroviral producer cells have been described elsewhere (7) and PlatA cells were a kind gift from Dr. Toshio Kitamura. The T2 cell line was from American Type Culture Collection and the HLA-A2⁺/Tyrosinase⁺ human melanoma cell lines 624 MEL and its autologous HLA-A2⁻ variant 624-28 MEL have been previously described (21). The hepatocellular carcinoma (HCC) cell line HepG2 was used with or without being transduced with a retrovirus encoding a HCV NS3₁₄₀₆₋₁₄₁₅ minigene. Briefly, synthetic oligonucleotides encoding the epitope and containing partial mutated Nco I restriction sites (5' primer - CATGAAGCTGGTCGCATTGGGCATCAATGCCGTGGCTGCTTATGG and 3' primer - CCATAAGCAGCCACGGCATTGATGCCCAATGCGACCAGCTTCATG) were annealed and the dsDNA product ligated into a retroviral vector, pMFG-EGFP. The recombinant vector was transiently transfected into PlatA packaging cells and the virus present in the culture supernatant used to transduce HepG2 cells. Transduced HepG2 cell cultures were FACS enriched for the EGFP expression and used as HCV NS3₁₄₀₆₋₁₄₁₅⁺ HCC cells. All cells were cultured at 37°C in a 7% CO₂ humidified incubator.

Culture Medium and Reagents

Tyrosinase₃₆₈₋₃₇₆, HCV NS₃₁₄₀₆₋₁₄₁₅ and control peptides (gp100₂₀₉₋₂₁₇, 210M and CMVpp65) were purchased from MP Systems (San Diego, CA). Culture medium for all cell lines was Iscove's Modified Dulbecco's Medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, West Sacramento, CA). Medium for T cells was XVIVO15 (Lonza, Walkersville, MD) supplemented with 5% heat-inactivated human AB serum (Gemini Bio-products), 2 mM L-glutamine (Cellgro, Herndon, VA), 20 mM HEPES (Cellgro) and 600 IU/ml interleukin (IL)-2 (Chiron, Emeryville, CA). Fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) conjugated mAbs against CD4, CD8 and CD34 and their respective isotype controls were purchased from BD Biosciences (San Jose, CA). PE-conjugated mAbs against human V β 12 was from Beckman Coulter (Miami, FL) and its IgG2a matched isotype control obtained from Biologend (San Diego, CA). 7AAD was purchased from Calbiochem (La Jolla, CA). PMA and ionomycin was purchased from Sigma Chemicals (St. Louis, MO).

Production of retrovirus for T cell transduction

PlatA cells were seeded on poly-D-Lysine-coated plates (BD Biosciences) in antibiotic free media. Next day, cells were co-transfected with the vesicular stomatitis virus envelope gene (Trono D, nature biotechnology, 1997) and the TIL 1383I TCR/CD34t or HCV cl 3 TCR/CD34t vectors, using Lipofectamine 2000 according to the manufacturers recommendations (Invitrogen). Two days later, virus present in the culture supernatant was harvested, filtered and used to transduce the GALV-pseudotyped MLV producing cell line PG13 in the presence of 8 μ g/ml of polybrene (Sigma). After two days, the transduced PG13 cells were harvested by trypsin (Cellgro), washed and reseeded for further propagation. The culture supernatant from homogenously CD34t⁺ PG13 cells were the source of retroviruses used to transduce activated human T cells.

Activation, expansion and transduction of primary human T cells

The procedure for polyclonal *in vitro* activation and expansion of T cells by anti-CD3 mAbs or Dynabeads has been previously described (9, 22, 23). Anti-CD3/anti-CD28-coated Dynabeads (Invitrogen) were added to T cells at a 1:3 cell:bead ratio and anti-CD3 mAbs used at a concentration of 50ng/ml. Cultures were maintained in media containing 300–600 IU/ml IL-2. Cells were transduced once or twice (at day 3 and/or 4 post activation) with the virus present in freshly harvested supernatants from nearly confluent CD34t⁺ PG13 cells. During each transduction, supernatants with TIL 1383I TCR/CD34t or HCV cl 3 TCR/CD34t retrovirus were pre-bound to retronectin (20 μ g/ml, Takara Bio Inc., Shiga, Japan) coated 6-well or 24-well non-tissue culture treated wells by centrifugation at 1200xg at 32°C for 90 minutes. The cells were then resuspended at 1 $\times$ 10⁶ cells/ml in more of the same retrovirus containing supernatant and supplemented with 600 IU/ml IL-2 before added to the pre-loaded wells. 1 ml was added to each well of 24 well plates and 9ml to each well of 6 well plates, before spinnoculation using the same conditions. Cells were incubated for 5 hours at 37 C before ~75% of the media was replaced with fresh media supplemented with 600 IU/ml IL-2 and cells then incubated overnight. This procedure was in some cases repeated the following day and cells maintained at cell concentrations between 0.75 $\times$ 10⁶ and 1.5 $\times$ 10⁶ cells/ml. One week after transduction, cells were harvested, and the anti-CD3/anti-CD28-coated magnetic beads removed by a magnet before the T cells were washed and cultured without stimulation for at least a day.

Flow cytometry and fluorescence-activated cell sorting

For phenotypical evaluation analytical flow cytometry analysis was used. Transduced and control cells were incubated in 30% human AB serum block subsequent unspecific mAb

binding. Staining with mixtures of fluorochrome-labeled mAbs, specific for e.g. CD34, V β 12 and CD8 or the respective isotype controls was performed for 30 minutes. After washing cells were stained with 7AAD and analyzed by flow cytometry or sorted by FACS. Flow cytometry for analysis of surface phenotypes was performed using an Accuri C6 flow cytometer and its designated software (Accuri Cytometers, Ann Arbor, MI). FACS was performed using a BD FACS Aria (BD Biosciences) and data subsequently analyzed and visualized using the FlowJo software (Tree Star Inc., Ashland, OR). Only the viable human lymphocytes, defined by their FSC/SSC profiles and their exclusion of 7AAD staining were analyzed for staining of mAbs. Four different fractions were isolated in the FACS: First, “Unsorted” cells were isolated irrespective of their CD34t expression level. Next, cells expressing three different distinct cell surface levels of CD34t (high, low but positive and negative) were concomitantly isolated. After culture over night, cells from each of the FACS fractions were analyzed by analytical flow cytometry as described above.

Enrichment of TCR transduced T cells using CD34-based stem cell isolation procedures

The transduced T cell cultures were enriched for CD34t⁺ cells using Dynal CD34 progenitor cell selection system (Invitrogen). Although the manufacturer’s protocol was developed for stem cell isolation, transduced T cells were successfully enriched by the standard methodology suggested. In brief, addition of anti-CD34 mAb-coated magnetic beads to the cells was followed by removal of the cells unable to bind the beads, collected as the CD34 negative fraction. The cells retained by the magnet were then dissociated from the beads and eluted as the CD34 positive fraction. Sham sorted cells, exposed to the same buffers and procedures but not the anti-CD34 mAb-coated magnetic beads, were used as the unsorted control fraction.

Co-culture experiments and ELISA

The functional responsiveness of the effector cells to antigen was determined by co-culturing 5×10^4 to 1×10^5 effector cells with 2.5×10^4 to 1×10^5 human tumor cells or peptide-pulsed T2 cells in a final volume of 0.2 ml in each round-bottom well of 96-well plates. T2 cells were pulsed with 1 μ g/ml of the cognate (Tyrosinase and HCV NS3 derived) HLA-A2 restricted peptides or irrelevant HLA-A2 restricted peptides(gp100_{209-217, 210M}, CMVpp65 or HCV NS3₁₄₀₆₋₁₄₁₅). HLA-A2⁺ human melanoma cells 624 MEL and the HCC cell line HepG2 transduced with a HCV NS3₁₄₀₆₋₁₄₁₅ minigene was used as the respective cognate target, while HLA-A2⁻ 624-28 MEL cells and untransduced HepG2 cells served as the negative controls. After 16 to 24 hours, culture supernatants were harvested and the concentrations of human IFN- γ released into the co-culture supernatant were estimated by measurement of the amounts of the cytokine in each well by sandwich ELISA as per manufacturer’s protocol (R&D Systems). Absorbance was measured at 450nm by a spectrophotometer and the amount of cytokine in the co-culture supernatants was calculated based on the absorbance of cytokine standards present in the assays. Where necessary the culture supernatants were diluted in culture media to be in the linear range of the assay.

Statistical analysis

Results of cytokine secretion by transduced T cells enriched using CD34-based isolation procedures are shown as mean $\pm$ standard deviation.

Results

T cells efficiently co-express CD34t and the TIL 1383I TCR β -chain upon transduction

We created new constructs linking the α -chain and the β -chain of the TIL 1383I TCR or the HCV cl 3 TCR to the 5’ end of CD34t via “self-cleaving” 2A sequences derived from

porcine teschovirus-1 (P2A) and *Thosea asigna* virus (T2A). Retroviruses encoding the resulting constructs were generated in CD34⁺ enriched PG13 retroviral producer cells using the SAMEN CMV retroviral backbone (Figure 1). Virus with 2A-linked TCR chains exhibited superior T cell transduction capacity compared to virus generated with the corresponding vectors using dual promoters (data not shown). The virus containing the TIL 1383I TCR/CD34t construct was first used to transduce normal PBL-derived T cells.

Activated PBL-derived T cells were transduced with the TIL 1383I TCR/CD34t retrovirus or culture media as sham control. Eight days later, each culture was stained with mAbs specific for CD34, Vβ12, or their respective isotype controls and CD4 or CD8. Based on CD34t expression, we find that individual experiments had 11–85% of the T cells transduced (data not shown). A detailed analysis of a typical experiment culture is shown in Figure 2. In this experiment, approximately 23% of the T cells transduced with the TIL 1383I TCR/CD34t retrovirus (Figures 2C,D) but not the sham control (Figure 2H) were transduced. Approximately 19.5% of the T cells expressed Vβ12⁺ TCRs (Figures 2B,D) on their cell surface. It should be noted that there is a population of T cells which naturally express Vβ12 that may not express the TIL 1383I TCR. Unlike other vectors, the inclusion of CD34t allowed us to distinguish these T cells expressing an endogenous Vβ12 TCR (Vβ12⁺ but CD34t⁻ cells) from T cells expressing the exogenous TIL 1383I TCR Vβ12-chain, as they are CD34t⁺/Vβ12⁺. When excluding the 1.9% of T cells which naturally express Vβ12 (based on the gate in Figure 2H), we estimate the percentage of T cells in the transduced culture that express the TIL 1383I TCR β-chain to be 17.5% (Figure 2D). Despite all of the chains being linked by 2A sequences and expressed as a single polypeptide, it is clear that expression of one chain does not always dictate the cell surface expression of the other chains. In this experiment, 7.2% of the CD34t⁺ T cells did not express Vβ12 and 1.7% of the Vβ12⁺ cells did not express CD34t. However, the higher the expression of either molecule on a cell the more probable it was to co-express the other one. Since 15.8% of the T cells stained for both CD34t and Vβ12 (Figure 2D), approximately 70% of the transduced bulk T cells expressed the TIL 1383I TCR β-chain. Conversely, 90% of the T cells expressing the TIL 1383I TCR β-chain expressed CD34t. These results indicate that CD34t is a good marker for transduced T cells expressing the TIL 1383I TCR on the cell surface.

The TIL 1383I TCR is a rare high affinity receptor that can engineer both CD4⁺ and CD8⁺ T cells to recognize tumor cells in an HLA-A2 restricted fashion (9). Therefore, it was important to determine if this TIL 1383I TCR/CD34t virus could efficiently transduce both CD4⁺ and CD8⁺ T cells. CD34t was efficiently expressed on both CD4⁺ (Figure 2E) and CD8⁺ (Figure 2F) T cells. However, the fraction of CD8⁺ T cells that expressed CD34t (28.2%) was higher than the fraction of CD4⁺ T cells that expressed CD34t (22.5%). In spite of this, the fraction of cells co-expressing both CD34t and Vβ12 was similar (16.2% vs. 15.7%) in the two T cell subsets. Thus, there was a higher degree of co-expression of the CD34t molecule and the TIL 1383I TCR β-chains in CD4⁺ T cells compared to CD8⁺ T cells. This observation was consistent in numerous transductions using T cells from several independent donors (data not shown).

It is well known that transgene expression is variable in any cell population. Another unique feature of the TIL 1383I TCR/CD34t vector is it also allow us to directly compare the expression levels of the endogenous and exogenous TCR β-chains more accurately. Notably, about half ((12.3%–1.9%)/19.4%) of the Vβ12⁺ T cells expressed the exogenous TIL 1383I TCRβ-chains at levels comparable to that exhibited by T cells carrying endogenous Vβ12⁺ TCRs (Figure 2B). The results were similar in the CD4⁺ and CD8⁺ T cell subsets (Figures 2E,F). These results indicate that transduced T cells with high cell surface levels of CD34t

can efficiently express foreign TCR's at a level comparable of an endogenous TCR in both T cell subsets.

CD34-based stem cell isolation procedures can enrich for engineered T cells

The critical application for vectors encoding CD34t is to use clinically relevant methods to rapidly enrich for transduced cells. Clinical grade CD34 selection systems have been developed for use in hematopoietic stem cell transplantation (24, 25). In order to determine if our CD34t containing vectors could be used for enriching for transduced cells, TIL 1383I TCR/CD34t transduced T cell cultures were purified once using anti-CD34 mAb coated immunomagnetic beads. T cells in the positive fraction, the negative fraction, and the unfractionated population were collected and assessed for CD34 and V β 12 expression and antigen recognition. Results from a representative experiment are shown in Figure 3. Flow cytometric analysis of the T cells in each fraction revealed an increased frequency of the cells expressing CD34t, from 15.5% in the unselected population to 76% in the positive fraction representing a 5-fold increase in transduced T cells (Figure 3A). The depleted fraction had 6.9% CD34t⁺ cells representing a greater than 50% depletion of transduced T cells. A more critical factor was the impact on TIL 1383I TCR expression. The unsorted population had 8.2% CD34t⁺/V β 12⁺ cells whereas the positive fraction had 42% CD34t⁺/V β 12⁺ cells representing a 5-fold enrichment (Figure 3A). The CD34t depleted fraction contained 2.8% CD34t⁺/V β 12⁺ cells representing a 65% reduction in TIL 1383I TCR expressing T cells. One additional point worth noting is the T cells expressing an endogenous V β 12⁺ TCR that were not transduced remained in the negative fraction and were not present in the CD34 enriched fraction. These results indicate that immunomagnetic beads designed for stem cell isolation can rapidly enrich for transduced T cells expressing the TIL 1383I TCR.

The ability of the T cells in each fraction to functionally respond to cognate antigen stimulation was also analyzed (Figure 3B). Effector cells from the positively sorted fraction secreted 3.5-fold more IFN- γ vs. T2 cells pulsed with cognate peptide, compared with unselected cells (Figure 3B). Importantly, the enrichment of reactivity against relevant human melanoma target cells was even greater with the positive fraction having approximately 5.5-fold enrichment relative to the unselected cells. As expected, the CD34t negative fraction had lost most of the reactivity vs. both the relevant targets compared with the unselected population. All effectors were responsive to polyclonal stimuli but exhibited minimal reactivity vs. non-cognate target cells (Figure 3B). Therefore, the 5-fold enrichment in the number of CD34t and TIL 1383I TCR expressing transduced T cells resulting from CD34 purification translated into a proportional increase in specific anti-tumor recognition.

CD34t-based fluorescence-activated cell sorting of transduced T cells

To determine the relative contribution of transduced T cells expressing high and low levels of the transgenes to the overall capacity of the cultures to recognize peptide pulsed target cells and melanoma tumors, we sorted the transduced cultures by FACS based on the level of CD34 expression. Primary human T cells were transduced once with TIL 1383I TCR/CD34t retrovirus, expanded for one week, then stained for CD34 expression and separated by FACS into four fractions. Results with the FACS isolated cells were similar to the CD34 bead enriched cells in that enrichment based on CD34 expression leads to enrichment of TIL 1383I TCR expressing cells. Results from a representative experiment are shown in Figure 4. The frequency of TIL 1383I TCR expressing T cells was 83% in the population sorted for high CD34 expression and 44% in the population sorted for low CD34 (Figure 4A). This represents a 7- and 3.5-fold enrichment respectively compared to the unsorted population. In terms of TIL 1383I TCR expression levels, the CD34⁺/V β 12⁺ T cells in the unsorted population had mean fluorescence intensity (MFI) of 5,140 for CD34 and 1,476 for V β 12,

an MFI of 10,384 for CD34 and 2,082 for V β 12 in the CD34 high sorted population, and an MFI of 1,077 for CD34 and 1,130 for V β 12 in the CD34 low sorted population. These results indicate that there is a strong relationship between the levels of CD34t and TIL 1383I TCR expression following selection based on CD34 expression.

The real question is how selection of transduced cells impacts on antigen recognition. The cells sorted for high CD34 expression exhibited approximately 12-fold increase in recognition of tyrosinase peptide pulsed T2 cells and more than 20-fold increase in reactivity against HLA-A2⁺ tumor cells compared to unsorted cells (Figure 4B). In contrast, T cells selected for low CD34 expression recognized cognate peptide loaded T2 cells more than twice as well as unsorted cells (Figure 4B) but failed to show enhanced specific recognition of tumor targets. As expected, the CD34⁺ cells lacked reactivity versus all targets. These results suggest that any method that can select for cells with the highest CD34t expression will also selectively enrich for cells with the highest expression level of the other linked transgenes.

CD34-based enrichment of the function of T cells transduced with a non-traceable transgene

All of the analysis has used the TIL 1383I TCR which can be detected using mAb against V β 12. Besides the availability of clinical grade reagents capable of purifying CD34 expressing cells without triggering signaling events, the CD34t selection technology enabled us to enrich for transgenes which can't be detected with mAbs. Another of our TCRs, which targets the HLA-A2 restricted 1406–1415 epitope from HCV NS3, is not bound by any of the commercially available TCR-subfamily specific mAbs (data not shown). Therefore, we applied our CD34t selection approach to HCV cl 3 TCR/CD34t retrovirus transduced T cells. Using the immunomagnetic beads, dramatic enrichment of both the fraction of cells transduced and the functionality of the resulting effectors was observed. Based on CD34t expression, we find that individual experiments had 8–42% of the T cells transduced (data not shown). Results from a representative experiment are shown in Figure 5. Compared to the unsorted population the enrichment using the progenitor cell selection system led to greater than 7-fold increase in the number of CD34t expressing cells in the positive fraction and approximately 50% depletion of CD34t expressing cells in the negative fraction (Figure 5A). There appears to be no selective bias against the enrichment or depletion of CD4⁺ or CD8⁺ T cells. The impact of CD34 selection on T cell function by each fraction was also analyzed (Figure 5B). Upon co-culture with T2 cells pulsed with cognate peptide, effector cells from the positively sorted fraction secreted about 4-fold more IFN- γ than the unsorted cells (Figure 5B). Importantly, the anti-tumor reactivity against HCV NS3₁₄₀₆₋₁₄₁₅⁺ HCC cells was enriched more than 6-fold. As expected, the CD34 negative fraction had reduced reactivity relative to the unsorted cells. All effectors were responsive to polyclonal stimuli but exhibited minimal reactivity vs. non-cognate target cells. These results confirm that our CD34t vectors can enrich for functional gene modified cells regardless of our ability to monitor expression of the transgene of interest.

Discussion

Adoptive transfer of TIL into patients rendered lymphopenic has revolutionized the efficacy of cancer immunotherapy (26, 27). Technical hurdles, in particular the difficulty to generate tumor reactive TIL cultures of sufficient numbers from most types of malignancies, have limited treatment to a small group of melanoma patients. Therapy with TCR-gene modified T cells circumvents the need to isolate tumor reactive T cells from patients by enabling fast and robust redirection of autologous normal T cells from any individual. Initial trials confirmed the feasibility and safety of this treatment modality (1, 2) and the promise to make adoptive T cell transfer more generally applicable gives it great potential.

Development of the technology to efficiently transduce and rapidly enrich redirected human T cells for prompt patient treatment is however needed to improve the efficacy of TCR-transduced effector cells. To address this need we developed a novel selection technology based on co-expression of a truncated version of the human CD34 molecule and the transgenes of interest. TCR-gene modified T cells could be readily enriched 5 to 7-fold, using clinically relevant methodology available for the isolation of CD34⁺ hematopoietic stem cells, and exhibited corresponding increases in the tumor specific reactivity. Furthermore, FACS mediated isolation revealed that optimizations of the beads-based enrichment protocols to preferentially isolate the effector cells expressing high levels of CD34t may further improve the functional reactivity of the resulting effector population and the power of this methodology. Implementation of this technology circumvents the critical problems currently preventing TCR-gene transfer from being a feasible and effective treatment option.

Only a fraction of the T cells exposed to transduction will express the introduced transgenes. Notably, studies in mouse models further suggest that the presence of additional non-transduced cells in the infused population may reduce the efficacy of adoptive cell transfer (11, 12). Therefore, it's desirable to enrich for the T cells that indeed have gained the desired reactivity before distributing the effector cells to patients. The gene product of neomycin resistance has often been used for selecting transduced cells. Regrettably, the protein encoded by neo^r is immunogenic in many humans and consequently the persistence and the clinical effect is poor (16). In contrast, human CD34 is an ideal selectable marker since it is not expressed by untransduced T cells but is non-immunogenic in all humans. Notably, many TCRs bind tetramer reagents or have an α - or β -chain that can be labeled with available antibodies. Thus, transduced cells could often instead be enriched by indirect bead capturing or FACS of cells labeled with tetramers or TCR-subfamily specific mAbs. However, the CD34t mediated enrichment is superior for several different reasons. Most importantly, direct binding of the TCR chains may trigger signaling events that negatively impact the future viability and reactivity of the effector cells (28). Since CD34t lacks signaling domain, this marker will not trigger cellular activation upon engagement by antibodies. Also, the technology developed for stem cell transplantation ensures that clinical grade reagents and apparatus to isolate also CD34⁺ T cells is readily available (24, 25). Finally, when the expression of the transgene can not be directly monitored in viable cells, as in the case of the HCV cl 3 TCR, the CD34t selection system provides a unique tool to enrich the engineered cells.

Clinical responses obtained by TIL therapy were associated with shorter *ex vivo* culture (29) and longer telomere length of infused TIL (13) that resulted in *in vivo* persistence (30). Generation of sufficient numbers of T cells for patient treatment usually necessitates extensive expansion and neomycin/G418 mediated enrichment of gene-modified cells requires prolonged *ex vivo* culture prior to patient infusion. Regrettably, this may be detrimental to T cell survival *in vivo* and thus the clinical efficacy (3, 13). *Ex vivo* engineering, expansion and enrichment of T cells can be accomplished in less than a week using the novel vectors. Therefore, the rapid and efficient CD34-based enrichment of redirected T cells will allow quicker treatment with short term cultured effector cells that may lead to better persistence and clinical efficacy.

Expression of CCR7 and/or CD62L divides human memory T cells into two functionally distinct subsets (31, 32). The CCR7⁻ T_{EM} cells display immediate effector function but poor persistence whereas CCR7⁺ T_{CM} cells display the reciprocal properties. The ability of the T_{CM} cells to both self-renew and differentiate into CCR7⁻ effector cells upon secondary stimulation make them the memory population of choice for adoptive immunotherapy against cancer. Importantly, the state of differentiation of effector cells also predicts their *in*

vivo efficacy (26). Expression of CD27 and CD28, markers of less differentiated T cells, on TIL was associated with anti-tumor activity in patients (33). For this reason a novel protocol to generate “young” TIL has recently been developed (34). In TCR-transgenic mice, naïve antigen specific T cells are readily available and upon appropriate activation these may mediate potent anti-tumor activity (35, 36). These cells represent the best fit antigen reactive cells possible, unfortunately there is no true human equivalent. However, in a lentiviral delivery system the CD34t-based selection methodology will likely allow rapid engineering, selection and infusion of naïve and resting T cells. The CD34t selection technology will thereby allow a type of adoptive cell therapy for humans which to date only can be achieved by genetic engineering of hematopoietic stem cells (37). The shorter *in vitro* exposure achieved compared to generating redirected T cells from hematopoietic stem cells *in vitro* and the lack of thymic selection necessary upon *in vivo* distribution of TCR-engineered hematopoietic progenitors make transduction and rapid CD34-based enrichment of naïve T cells an attractive alternative.

The clinically available CD34-based selection system has been exploited for enrichment of gene-modified T cells before (38). However, CD34 expression on human T cells have only been described in the context of fusion proteins, consisting of variants of CD34 and herpes simplex virus thymidine kinase (CD34/HSV-tk) that confer sensitivity to ganciclovir (GCV). These constructs were developed for suicide gene therapy of infused donor T cells as safety mechanisms against graft-versus-host disease following allogeneic hematopoietic cell transplantation (HCT) (39, 40). The usefulness of this strategy was hampered by the development of CD34⁺ GCV-resistant T cells subjected to the selective pressure of GCV (41). The presence of cells that express the selectable marker in absence of the other transgenes may jeopardize the safety of suicide gene technology. When CD34 expression is used as a selectable cell surface marker to enrich TCR-transduced T cells there is no selective pressure for the generation of cells positive for one transgene but not the other. More importantly, even if such cells arise it will in this case not constitute a major problem. The application of CD34 as an independent selectable marker instead of a part of a fusion protein also allows enrichment for a much wider range of transgenes, including cell surface receptors. Thus, while previously only useful for selection of allogeneic effector cells for treatment of HCT patients the novel vectors enable enrichment of effector cells for virtually any transgene.

Various alternative strategies have been developed to improve the expression level, functional capacity and pairing of introduced TCRs. These include codon optimization, introduction of cysteines into the constant regions of the TCR, introduction of mouse constant TCR regions and modification of N-glycosylation sites (42–46). Since the endogenous and exogenous TCRs are known to compete for cell surface expression, it is indeed crucial to promote preferential expression of the retrovirally introduced TCR to ensure its cell surface expression and function (47). Compared to other strategies developed the CD34t-based selection technology is more generally applicable as any human TCR can be easily cloned into the now available vectors through the use of a conserved *StuI* restriction site in the constant region of human TCR β -chains (Figure 1B). Compared to some of the other strategies it also decreases the risk of that the redirected cells are immunogenic in the patient. Moreover, the use of the CD34t selection system does not prevent concomitant employment of other strategies and its combination with codon optimization, introduction of cysteines and modification of N-glycosylation sites in the constant regions of the TCR are warranted in future studies.

The use of vectors co-expressing CD34t molecules and TCRs also enable improved immune monitoring. Since CD34 is not expressed by normal T cells, it is possible to trace genetically altered T cells from human and animal tissues based on their CD34t expression. Although,

persistence of infused T cells previously have been traced in peripheral blood samples by TCR specific PCR (48) there is a huge advantage to instead apply cell surface expression of CD34t to visualize these cells in patient samples. It will allow more accurate quantification and localization as well as enable *in situ* immunohistochemistry and immediate functional evaluation and isolation of viable effector cells *ex vivo*. This additional benefit of the CD34t enrichment technology will likely enhance our ability to understand the factors that influence the localization, persistence and function of adoptively transferred T cells.

In conclusion, we provide proof of concept for a novel enrichment technology, which now can be used to potentiate a wide variety of vectors and expression products that normally would be hard to select for. Thus, we assert that the viral vectors having the potent and clinically compatible CD34-based enrichment technology will not only benefit the TCR gene therapy field, but will help make substantial advancements in cellular engineering in general.

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Abbreviations used

CTL	cytolytic T lymphocytes
PBMC	peripheral blood mononuclear cells
TCR	T cell receptor
IFN	Interferon
FACS	fluorescence-activated cell sorting
ELISA	enzyme-linked immunosorbent assay

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Figure 1. Retroviral constructs

A) Linkage of the α -chain and β -chain of the respective TCR via self-cleaving P2A molecules using PCR generated 1383I α P2A β and HCVcl3 α P2A β . The hybrid 5' LTR, containing the human cytomegalovirus enhancer and promoter fused to the MMLV 5' LTR drive the TCR expression upon transduction using the resulting SAMEN-1383I α P2A β and SAMEN-HCVcl3 α P2A β . **B)** The IRES/neo^r cassettes were replaced with a truncated version of the CD34 molecule (CD34t), linked to the β -chain of the respective TCR via self-cleaving T2A molecules by restriction digestion with *Stu*I and *Bam*HI. The resulting vectors were designated TIL 1383I TCR/CD34t and HCV cl 3TCR/CD34t, respectively. Ψ , packaging signal; SD, splice donor; SA, splice acceptor.

Figure 2. The TIL 1383I TCR/CD34t retrovirus mediate efficient co-expression of the CD34t molecule and the TIL 1383I TCR β -chain on the cell surface of primary human T cells

Healthy donor bulk PBL were activated with anti-CD3/anti-CD28-coated magnetic beads, transduced twice with TIL 1383I TCR/CD34t retrovirus or sham transduced and expanded as described in materials and methods. After one week, cells were harvested, the beads removed by a magnet and cells cultured overnight. Retrovirus and sham transduced cells were then harvested, stained with mAbs specific for CD34, V β 12 and CD4 or CD8 or the respective isotype controls before exposed to 7AAD and analyzed by flow cytometry. All samples were first gated as viable lymphocytes in FSC/SSC and as 7AAD negative cells. In the lower panels, cells were further gated on all cells, CD4⁺ cells or CD8⁺ cells respectively. All plots show the relative logarithmic immunofluorescence intensities of FL2 versus FL1. The data shown is from one representative experiment of five with similar results.

Figure 3. Enrichment of redirected T cells using CD34-based stem cell isolation procedures results in dramatically enhanced antigen specific tumor targeting

T cells transduced with TIL 1383I TCR/CD34t retrovirus (as described in Figure 2) were separated in two fractions using CD34 progenitor cell selection system. After addition of anti-CD34 mAb-coated magnetic beads the cells unable to bind the beads, and thus to be retained by the magnet, were collected as a “34t-Negative” fraction. The remaining cells were then dissociated from the beads and in the presence of a magnet eluted as a “34t-Positive” fraction. Sham sorted cells, exposed to the same buffers and procedures but not the actual anti-CD34 mAb-coated magnetic beads, were used as the “Unsorted” control fraction. **A)** Cells from each of the three fractions were then stained with mAbs specific for CD8, Vβ12 and CD34 or the respective isotype controls before exposed to 7AAD and analyzed by flow cytometry. All samples were first gated as lymphocytes in FSC/SSC and as 7AAD negative cells. All plots show the relative logarithmic immunofluorescence intensities of FL2 versus FL4. **B)** In parallel, 5×10^4 cells from each of the three fractions were co-cultured over night with 5×10^4 peptide-pulsed, irrelevant gp100:209M or cognate tyrosinase:YMD, T2 cells or HLA-A2 positive melanoma 624 MEL cells or matched HLA-A2 negative 624-28 MEL cells. The amount of human IFN-γ released into the co-culture supernatant was measured by sandwich ELISA and the respective concentrations of cytokine present in wells containing various combinations of effector and target cells was estimated. Bars represent the mean and error bars the standard deviation between duplicate cultures. The data shown is from one representative experiment of three with similar results.

Figure 4. Fluorescence-activated cell sorting of transduced T cells into fractions expressing distinct cell surface levels of CD34t

Healthy donor PBL-derived T cells were activated with anti-CD3/anti-CD28-coated magnetic beads and transduced once with TIL 1383I TCR/CD34t retrovirus and expanded for one week. Cells were then harvested and the beads carefully removed by a magnet and cells frozen. Upon thawing cells were stained with anti-CD34-FITC mAb and 7AAD and sorted by FACS. Four different fractions of 7AAD negative cells were isolated. Cells isolated irrespective of their CD34t expression was used as the “Unsorted” control. In addition, the CD34t⁺ cells were divided down the middle in two equal portions. The cells sorted as the half having highest CD34t expression was designated the “CD34t-High” fraction and the remaining CD34t⁺ cells was defined as the “CD34t-Low” fraction. Finally, CD34t⁻ cells was isolated as the “CD34-Negative” fraction. **A)** After culture over night, cells from each of the FACS fractions were harvested and stained with mAbs specific for CD34, Vβ12 and CD8 before exposed to 7AAD and analyzed by flow cytometry. All samples were first gated as lymphocytes in FSC/SSC and as 7AAD negative cells. All plots show the relative logarithmic immunofluorescence intensities of FL2 versus FL1. **B)** In parallel, 5×10^4 cells from each of the sorted fractions were co-cultured over night with 2.5×10^4 peptide-pulsed, irrelevant HCV NS3₁₄₀₆₋₁₄₁₅ or cognate tyrosinase₃₆₈₋₃₇₆, T2 cells or 5×10^4 HLA-A2 positive melanoma 624 MEL cells or HLA-A2 negative 624-28 MEL cells. The amount of human IFN-γ released into the co-culture supernatant was measured by sandwich ELISA and the respective concentrations of cytokine present in wells containing various combinations of effector and target cells was estimated. Bars represent the mean and

error bars the standard deviation between duplicate cultures. The data shown is from one representative experiment of two with similar results.

Figure 5. CD34-based enrichment of the function of redirected T cells transduced with a non-traceable transgene

Bulk PBL were activated with anti-CD3/anti-CD28-coated magnetic beads and transduced once with HCV cl 3 TCR/CD34t retrovirus. One week later cells were harvested and all the beads removed by a magnet. T cells were then separated into two fractions using CD34 progenitor cell selection system. The cells unable to bind the anti-CD34 mAb-coated magnetic beads were collected as a “34t-Negative” fraction. The remaining cells were dissociated from the beads and in the presence of a magnet eluted as the “34t-Positive” fraction. Sham sorted cells, were used as the “Unsorted” control fraction. A) Cells from each of the three fractions were stained with mAbs specific for CD34 or its isotype control and CD8 and 7AAD. Flow cytometric analysis of the viable primary human T lymphocytes, defined by their FSC/SSC profiles and 7AAD negativity, is shown. All plots show the relative logarithmic immunofluorescence intensities of FL4 versus FL1. B) 1×10^5 cells from each of the three fractions were co-cultured over night with 1×10^5 target cells. Peptide-pulsed, irrelevant CMV_{pp65} or cognate HCV NS3₁₄₀₆₋₁₄₁₅, T2 cells or the HLA-A2 positive hepatocellular carcinoma cell line HepG2, with or without a HCV NS3₁₄₀₆₋₁₄₁₅ minigene. The amount of human IFN- γ released into the co-culture supernatant was measured by sandwich ELISA and the respective concentrations of cytokine present in wells containing various combinations of effector and target cells was estimated. Bars represent

the mean and error bars the standard deviation between duplicate cultures. The data shown is from one representative experiment of five with similar results.