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ORIGINAL ARTICLE

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. Although promising, the therapy is still largely ineffective, as most patients did not 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. We developed novel and generally applicable retroviral vectors that allow rapid and efficient selection of T cells transduced with human TCRs. These vectors encode two TCR chains and a truncated CD34 molecule (CD34t) in a single mRNA transcript. Transduced T cells were characterized and the effects of CD34-based enrichment of redirected T cells were evaluated. Both $CD8^+$ and $CD4^+$ T cells could be transduced and efficiently co-expressed all introduced transgenes on their surface. Importantly, more than fivefold enrichment of both the frequency of transduced cells and the specific anti-tumor reactivity of the effector population could be achieved by magnetic beads-based enrichment procedures readily available for clinical grade hematopoietic stem cell isolation. This CD34-based enrichment technology will improve the feasibility of adoptive transfer of clinically relevant effectors. In addition to their enhanced tumor recognition, the enriched redirected T cells may also show superior reactivity and persistence in vivo due to the high purity of transduced cells and the shortened ex vivo culture.

Keywords TCR · Transduction · Selection · Immunotherapy · Melanoma

Abbreviations

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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Introduction

Adoptive transfer of autologous tumor infiltrating lymphocytes (TIL) to melanoma patients previously rendered

lymphopenic has revolutionized the efficacy of cancer immunotherapy as most subjects experience objective clinical responses [1–3]. While providing an important proof of concept, technical hurdles including the difficulty to generate sufficient TIL from most types of malignancies, has prevented this treatment regimen from moving beyond experimental phase. T cell specificity can be redirected by expression of exogenous T cell receptor (TCR) genes, using retroviral vectors (RV) [4–7]. Thereby, autologous tumor reactive effectors can readily and quickly be generated regardless of patients' capacity to spontaneously mount immune responses. The first reports using TCR gene-modified T cells in patients confirmed the feasibility and safety of this treatment and described objective clinical responses [8–10]. However, transduced PBL-derived T cells were less effective than TIL.

Several factors may prevent TCR gene-modified T cells from being efficacious. In the absence of selection, most infused cells failed to express the introduced TCR and thus lacked tumor antigen reactivity [9]. Importantly, pre-clinical in vivo studies further indicate that contaminating non-transduced cells may actively impair the efficacy of redirected cells [11, 12]. The extensive ex vivo culture employed may also be detrimental to the therapeutic efficacy of the T cells [13]. Novel technology to rapidly and efficiently enrich clinical grade TCR transduced T cells for prompt patient treatment, would circumvent these obstacles, and is needed to improve the efficacy of therapy.

Enrichment for transduced T cells requires antibiotic-based selection, e.g. by co-expression of transgenes with bacterial neomycin phosphotransferase enzyme (neo^r), which confers resistance to the neomycin analogue G418. Regrettably, neo^r and other selection markers currently used (e.g. enhanced green and yellow fluorescent proteins) are often immunogenic in vivo [14, 15]. This may severely limit the persistence of the effector cells, which was described to correlate with their clinical effectiveness [16]. Therefore, novel means of marking and enriching genetically altered T cells are needed to provide patients with non-immunogenic and non-exhausted gene-modified T cells.

By co-expressing a truncated version of the human CD34 molecule (CD34t) with an introduced TCR, we have developed an improved type of enrichment technology for redirected cells. TCR transduced T cells were enriched five to sevenfold based on their surface expression of CD34t using methodology readily available in the clinic for isolation of clinical grade $CD34^+$ hematopoietic stem cells for transplantation. Importantly, the enriched T cells exhibited a corresponding five to sixfold increase in specific anti-tumor reactivity.

Materials and methods

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

We incorporated GSG linked 2A ribosomal slippage sequence from porcine teschovirus-1 (P2A) [17], downstream of TCR α-chains by polymerase chain reaction (PCR), using TCR specific Vα 5′ primers and a common 3′ primer (GGGCCCAGGACCGGGGTTCTCCTCCACGTC TCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCT CCGGATCCACCGGTGCTGGACCACAGCCGCAG). Similarly, a complementary sequence was introduced upstream of the TCR β-chains using Cβ1 or Cβ2 specific 3′ primers and TCR specific Vβ 5′ primers (GACGTGG AGGAGAACCCCGGTCCTGGGCCCGGCACAAGGTT GTTCTTCTATGTG for TIL 1383I TCR and GCAGG AGACGTGGAGGAGAACCCCGGTCCTGGGCCCACT ATCAGGCTCCTCTGCTACATG for HCV cl 3 TCR). The products were used as templates in another PCR, using the same Vα 5′ primers and the Cβ1 or Cβ2 primers, respectively, creating two different P2A-linked full-length TCRαP2Aβ fusion cDNA molecules. Ligation into pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA) and transformation of *Escherichia coli* TOP 10 competent bacteria (Invitrogen) generated ampicillin-resistant bacterial clones, screened for presence of cDNAs by PCR. Plasmids were extracted with miniprep kit (Qiagen, Valencia, CA, USA), sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed with ABI Prism 310 Genetic Analyzer (Applied Biosystems). Mutations and undesired internal restriction sites were removed by site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, USA). Resulting constructs were denoted 1383IαP2Aβ and HCVcl3αP2Aβ.

The internal SRα-promoter of the SAMEN-CMV/SRα RV vector [18] was replaced by the excised 1383IαP2Aβ or HCVcl3αP2Aβ cDNA. 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 RV denoted SAMEN-1383IαP2Aβ and SAMEN-HCVcl3αP2Aβ (Fig. 1a).

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

The human CD34 molecule contains a signaling domain. To prevent the selection marker from influencing the function of transduced cells a truncated version of the CD34 molecule (CD34t, lacking the intracellular region of the protein) was cloned by reverse transcriptase PCR.

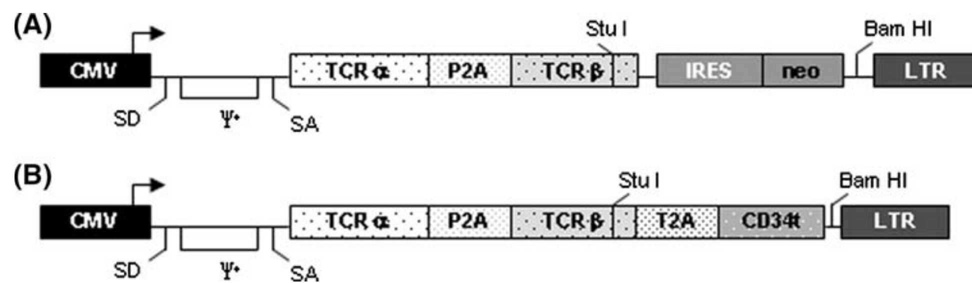

Fig. 1 Retroviral constructs. **a** The α- and β-chains of each TCR were linked by the P2A sequence using PCR. Cloning behind the hybrid 5′ LTR, containing the human cytomegalovirus enhancer and promoter fused to MMLV 5′ LTR, created SAMEN-1383IαP2Aβ and SAMEN-HCVcl3αP2Aβ. **b** IRES/neo^r cassettes were replaced with a truncated version of the CD34 molecule (CD34t) linked to the β-chain of the respective TCR via the T2A sequence. The resulting vectors were designated TIL 1383I TCR/CD34t and HCV cl 3 TCR/CD34t, respectively. *LTR* long terminal repeat, *ψ* packaging signal, *SD* splice donor, *SA* splice acceptor, *α* alpha chain, *β* beta chain

The 5′ end of CD34t was fused to GSG linked 2A ribosomal slippage sequence from *Thosea asigna* virus (T2A) [17] and the 3′ end of the respective TCR's β-chain using PCR. Two partially overlapping 5′ primers were used for each TCR, (GCTGGTCAGCGCCCTTGTGTTGATGGCAATGGTCAAGAGAAAGGATTTCGAATTCGGCTCAGGCGAGGGCAGAGGCAGTCTGCTAACATGCGGTGATGTCGAAGAAAATCCTGGCCCACCGCGGGGCTGGACCGCGC and GCCGAGGCCTGGGGTAGAGCAGACTGTGGCTTTACCTCGGTGTCCTACCAGCAAGGGGTCCTGTCTGCCACCATCCTCTATGAGATCCTGCTAGGGAAGGCCACCCTGTATGCTGTGCTGGTCAGCGCCCTTGTGTTG) compatible with Cβ1 of the TIL 1383I TCR β-chain and (GGTCAGTGCCCTCGTGCTGATGGCCATGGTCAAGAGAAAGGATTCCAGAGGCGAATTCGGCTCAGGCGAGGGCAGAGGCAGTCTGCTAACATGCGGTGATGTCGAAGAAAATCCTGGCCCACCGCGGGGCTGGACCGCGC and GCCGAGGCCTGGGGTAGAGCAGACTGTGGCTTCACCTCCGAGTCTTACCAGCAAGGGGTCCTGTCTGCCACCATCCTCTATGAGATCTTGCTAGGGAAGGCCACCTTGTATGCCGTGCTGGTCAGTGCCCTCGTGCTGATG) containing part of Cβ2 of the HCV cl 3 TCR β-chain. The common 3′ primer contained a *Bam*HI site (AAATCTAGAGTCGACGCGGCCGCGGATCCTCATGGTTCTAGTTCCAGCCTTTC). Obtained PCR products were cloned, sequenced, and undesired restriction sites and mutations removed by site-directed mutagenesis. To allow CD34-based selection of TCR transduced cells, we introduced the respective CD34t fragment into the SAMEN-1383IαP2Aβ and SAMEN-HCVcl3αP2Aβ vectors (Fig. 1b). Using a conserved *Stu*I site in the constant region of the β-chains of human TCRs and *Bam*HI sites we replaced the internal ribosomal entry site (IRES)/neo^r segments to create the final RV (TIL 1383I TCR/CD34t and HCV cl 3 TCR/CD34t), expressing single mRNA transcripts encoding three functional proteins.

Cells

Leukopheresis from healthy donors, obtained following informed consent, were from Research Blood Components, LLC (Brighton, MA, USA). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with lymphocyte separation medium (Mediatech Inc., Herndon, VA, USA) and frozen (100×10^6 cells/ml). PBL were isolated after plastic adherence depletion of PBMC. PG13 gibbon ape leukemia virus packaging cells were described elsewhere [4], PlatA cells were a kind gift from Dr. Toshio Kitamura (Department of Hematopoietic Factors, Institute of Medical Science, University of Tokyo, Tokyo, Japan), and T2 cells were from ATCC. HLA-A2$^+$/Tyrosinase$^+$ human melanoma cell line 624 MEL and an autologous HLA-A2$^-$ variant (624-28 MEL) were described [19]. Hepatocellular carcinoma (HCC) cell line HepG2 was used untransduced or transduced with retrovirus encoding hepatitis C virus (HCV) $NS3_{1406–1415}$ minigene. All cells were cultured at 37°C in a 7% CO_2 humidified incubator.

Culture medium and reagents

$Tyrosinase_{368–376}$, HCV $NS3_{1406–1415}$ and control peptides ($gp100_{209–217, 210M}$ and CMVpp65) were from MP Systems (San Diego, CA, USA). Cell lines were cultured in Iscove's Modified Dulbecco's Medium (GIBCO, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, West Sacramento, CA, USA). T cell medium was XVIVO15 (Lonza, Walkersville, MD, USA) with 5% heat-inactivated human AB serum (Gemini Bio-products), 2 mM L-glutamine (Cellgro, Herndon, VA, USA), 20 mM HEPES (Cellgro) and 300–600 IU/ml interleukin (IL)-2 (Chiron, Emeryville, CA, USA). Fluorochrome-conjugated mAbs against CD4, CD8, CD34, and

isotype controls were from BD Biosciences (San Jose, CA, USA). PE-conjugated anti-human-Vβ12 mAb was from Beckman Coulter (Miami, FL, USA) and IgG2a isotype control from Biolegend (San Diego, CA, USA). 7AAD was from Calbiochem (La Jolla, CA, USA) and PMA and ionomycin were from Sigma Chemicals (St. Louis, MO, USA).

Retrovirus production

PlatA cells were co-transfected with vesicular stomatitis virus envelope gene and TIL 1383I TCR/CD34t or HCV cl 3 TCR/CD34t vectors, using Lipofectamine 2000 (Invitrogen). Two days later, retroviral supernatants were harvested, filtered, supplemented with 10 µg/ml of protamine sulfate (Sigma), and used to transduce PG13 cells. Retroviral supernatants from CD34t$^+$ PG13 cells were used to transduce activated human T cells.

Activation, expansion, and transduction of primary human T cells

Polyclonal in vitro activation and expansion of T cells by anti-CD3 mAbs or Dynabeads was previously described [6, 20]. 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 50 ng/ml. T cell concentration was 0.5×10^6 to 1.5×10^6 cells/ml and cells were transduced once or twice (day 3 and/or 4 post activation). Retroviruses were pre-bound to retronectin-coated (20 µg/ml, Takara Bio Inc., Shiga, Japan) non-tissue culture treated 6- or 24-well plates by centrifugation. T cells were resuspended (1×10^6 cells/ml) in media or fresh retrovirus supernatant with 300–600 IU/ml IL-2 and spinoculated (1200–2000$\times g$, 32°C, 2 h) in the respective pre-loaded wells. Dynabeads were removed with magnet 1 week after transduction and T cells cultured without stimulation overnight.

Flow cytometry and fluorescence-activated cell sorting

Transduced and control cells were blocked in 30% human AB-serum. Staining with combinations of fluorochrome-labeled mAbs (e.g. anti-CD34, anti-Vβ12 and anti-CD8) was followed by washes, staining with 7AAD and analysis by flow cytometry on Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) or BD FACSCanto (BD Biosciences) and FlowJo software (Tree Star Inc., Ashland, OR, USA). Only viable lymphocytes (7AAD$^-$) were analyzed for mAbs staining. FACS was performed on a BD FACSAria (BD Biosciences) and four fractions were sorted (CD34t independent, CD34t^{high}, CD34t^{low} and CD34t$^-$), cultured overnight and analyzed by flow cytometry.

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

Transduced cell cultures were enriched for CD34t$^+$ cells using Dynal CD34 progenitor cell selection system (Invitrogen), according to the manufacturer's protocol developed for stem cell isolation. Briefly, addition of anti-CD34 mAb-coated magnetic beads was followed by removal of unbound cells (CD34 negative fraction). Cells retained by the magnet were dissociated from beads and eluted as the CD34 positive fraction. Sham sorted cells (control fraction) were not exposed to beads.

Cytokine release assays

The functional responsiveness to antigen was determined by co-culturing $0.5–1 \times 10^5$ effector cells with $0.25–1 \times 10^5$ human tumor cells or T2 cells pulsed with HLA-A2 restricted peptides (1 µg/ml of cognate, tyrosinase- and HCV NS3-derived, or irrelevant, $gp100_{209–217,210M}$, CMVpp65 or HCV $NS3_{1406–1415}$) in a final volume of 0.2 ml in round-bottom 96-well plates. 624 MEL and HepG2 cells transduced with HCV $NS3_{1406–1415}$ minigene were cognate tumor targets, while 624-28 MEL and untransduced HepG2 cells served as negative controls. After 16–24 h, concentrations of human IFN-γ in culture supernatants were estimated by sandwich ELISA (R&D Systems). Where necessary, culture supernatants were diluted to the linear range of the cytokine standards of each assay.

Statistical analysis

Results are presented by graphical displays. Density or dot plots are used to visualize phenotypical cell characteristics by direct presentation of flow cytometry data. The mean and standard deviation for each set of duplicate experimental cultures in functional assays were calculated using Microsoft Office Excel 2003 version 11. Results are depicted by histograms, showing mean ± standard deviation of cytokine secretion, in each group of effectors for the respective target cell line.

Results

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

We created new SAMEN-CMV RV linking the α- and β-chains of the TIL 1383I TCR or the HCV cl 3 TCR to CD34t via 2A sequences (Fig. 1). Retroviral vectors (RV) with 2A-linked TCR chains exhibited superior T cell transduction and/or expression capacity compared to the

corresponding RV using dual promoters (data not shown). Primary human T cells transduced with TCR encoding retrovirus (TIL 1383I TCR/CD34t or SAMEN-1383IαP2Aβ) or sham control (culture media) were expanded for 8 days and then stained with mAbs specific for CD34, Vβ12, or their respective isotype controls and CD4 or CD8. As expected, a fraction of the T cells exposed to SAMEN-1383IαP2Aβ retrovirus expressed the TIL 1383I TCR β-chain, but did not stain with anti-CD34 mAbs (data not shown). The transduction efficacy of the TIL 1383I TCR/CD34t retrovirus was determined by the frequency of CD34t-expressing cells. Depending on the specific circumstances for each transduction and the blood donor used, individual experiments had 11–85% of the T cells transduced (data not shown). In a typical experiment, approximately 23% of the T cells were transduced with the TIL 1383I TCR/CD34t retrovirus (Fig. 2c, d) but not with the sham control (Fig. 2h). About 19.5% of the T cells expressed $V\beta12^+$ TCRs (Figs. 2b, d) on their cell surface. Notably, there is a population of T cells expressing endogenous Vβ12 TCR (Fig. 2h). Unlike other vectors, the inclusion of CD34t allowed us to distinguish these T cells ($V\beta12^+$ but $CD34t^-$ cells) from T cells expressing exogenous TIL 1383I TCR Vβ12-chain ($CD34t^+/V\beta12^+$). When excluding the 1.9% of T cells that naturally express Vβ12 (based on the gate in Fig. 2h), we estimate the percentage of T cells expressing TIL 1383I TCR β-chain to be 17.5% (Fig. 2d). In spite of all chains being linked by 2A sequences and the polypeptides translated from a single mRNA molecule, it is clear that expression of one protein does not always dictate the cell surface expression of the others. In this experiment, 7.2% of $CD34t^+$ T cells did not express Vβ12 and 1.7% of TIL 1383I TCR $V\beta12^+$ cells did not express CD34t. Although not absolute, there was a strong correlation between the expression of CD34t and the TIL 1383I TCR. Since 15.8% of T cells stained for both CD34t and Vβ12 (Fig. 2d), approximately 70% of the transduced bulk T cells expressed TIL 1383I TCR β-chain. Conversely, 90% of the T cells expressing TIL 1383I TCR β-chain expressed CD34t. These results are representative for more than five individual experiments and indicate that CD34t is a good marker for transduced T cells expressing the TIL 1383I TCR on their 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 [6]. 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 $CD4^+$ (22.5%, Fig. 2e) and $CD8^+$ (28.2%, Fig. 2f) T cells. Since the fraction of cells expressing both CD34t and Vβ12 was similar (15.7 vs. 16.2%), there was a higher degree of co-expression of transgenes 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).

The unique ability to distinguish T cells expressing endogenous Vβ12-chains and the TIL 1383I TCR β-chain

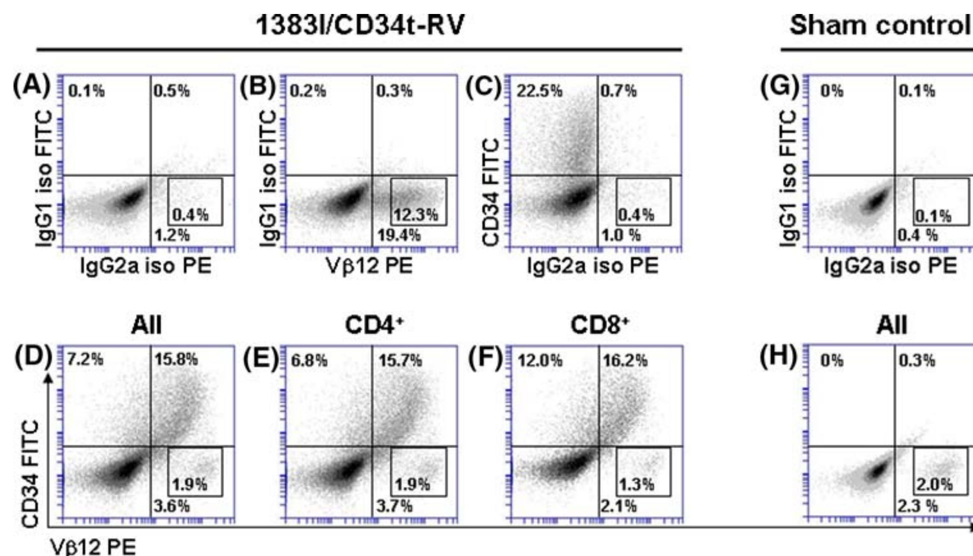

Fig. 2 TIL 1383I TCR/CD34t retrovirus mediates efficient co-expression of CD34t and TIL 1383I TCR β-chain on the cell surface of transduced primary human T cells. Activated bulk PBL were transduced twice with TIL 1383I TCR/CD34t retrovirus or sham transduced and expanded. After 1 week, cells were harvested, beads removed by a magnet and cells cultured overnight. The following day, cells were stained with mAbs specific for CD34, Vβ12 and CD4 or CD8 or the respective isotype controls before exposed to 7AAD and analyzed by flow cytometry. All plots show the relative logarithmic immunofluorescence intensities of FL2 versus FL1 and depict cells gated as viable lymphocytes, $CD4^+$ cells or $CD8^+$ cells, respectively. The data shown is from one representative experiment of five

further allows us to directly compare the expression levels of the endogenous and exogenous TCR β-chains more accurately. More than half [(12.3–1.9%)/17.5%] the T cells expressing TIL 1383I TCR β-chain exhibit similar cell surface expression levels of Vβ12 as T cells with endogenous V$\beta 12^+$ TCRs (Fig. 2b, d). The results were similar for $CD4^+$ and $CD8^+$ T cell subsets (Fig. 2e, f) and representative for more than five individual experiments. These results indicate that transduced T cells with high cell surface levels of CD34t can efficiently express introduced TCRs at levels comparable of endogenous TCRs in both T cell subsets.

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

The critical application for this novel RV is to use clinically relevant CD34 selection systems, readily available for hematopoietic stem cell transplantation, to rapidly enrich redirected cells. To determine if our TIL 1383I TCR/CD34t RV could be used to enrich transduced T cell cultures for redirected effectors by the clinically available methodology we evaluated purification using anti-CD34 mAb-coated immunomagentic beads. T cells in the positive fraction, the negative fraction, and the unfractionated population were assessed for CD34t and Vβ12 expression and antigen recognition. Results from a representative experiment are shown in Fig. 3. Flow cytometry analysis of the T cells in each fraction revealed a fivefold increase in the frequency of transduced cells, from 15.5% in the unselected population to 76% in the positive fraction (Fig. 3a). The negative fraction had a depletion of transduced T cells greater than 50%. More importantly, the fraction of T cells expressing TIL 1383I TCR β-chain ($CD34t^+$/V$\beta 12^+$) was increased fivefold from 8.2% in the unsorted population to 42% in the positive fraction (Fig. 3a). The CD34t negative fraction exhibited a 65% reduction in the frequency of T cells expressing TIL 1383I TCR β-chain. Moreover, untransduced T cells expressing endogenous V$\beta 12^+$ TCRs were not present in the CD34 enriched fraction, but remained in the CD34 negative fraction. These results indicate that immunomagentic beads designed for stem cell isolation can rapidly enrich for transduced T cells expressing TIL 1383I TCR.

The ability of the T cells in each fraction to respond to cognate antigen stimulation was also analyzed. Effector cells from the positive fraction secreted 3.5-fold more IFN-γ versus T2 cells pulsed with cognate peptide, compared to unselected cells (Fig. 3b). Importantly, the enrichment of reactivity against relevant melanoma cells was even greater, approximately 5.5-fold, in the positive fraction relative to unselected cells. As expected, the CD34t negative fraction had lost most of the reactivity versus both relevant targets, compared to the unselected population. All effectors were responsive to polyclonal stimuli and exhibited minimal reactivity versus non-cognate target cells (Fig. 3b). Therefore, the fivefold 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 reactivity.

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 reactivity, we subjected transduced T cell cultures to CD34-based FACS. TIL 1383I TCR/CD34t retrovirus transduced T cells were expanded for 1 week before stained with anti-CD34 mAbs and separated by FACS into four fractions. Results from a representative experiment are shown in Fig. 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 levels of CD34 (Fig. 4a). This represents a 7- and 3.5-fold enrichment, respectively, compared to the unsorted population. In terms of expression levels, the $CD34^+$/V$\beta 12^+$ T cells in the unsorted population had mean fluorescence intensity (MFI) of 5,140 for CD34 and 1,476 for Vβ12, an MFI of 10,384 for CD34 and 2,082 for Vβ12 in the $CD34^{high}$ sorted population, and an MFI of 1,077 for CD34 and 1,130 for Vβ12 in the $CD34^{low}$ sorted population (data not shown). These results indicate a strong relationship between expression levels of CD34t and TIL 1383I TCR, and that CD34-based purification of T cells with the highest levels of CD34t expression will yield the highest levels also of other linked transgenes.

The real question is how selection of transduced cells affects antigen recognition. $CD34^{high}$ sorted T cells 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 (Fig. 4b). $CD34^{low}$ sorted T cells recognized cognate peptide loaded T2 cells more than twice as well as unsorted cells but failed to show enhanced specific recognition of tumor targets (Fig. 4b). As expected, the $CD34t^-$ cells lacked reactivity versus all targets. These results suggest that optimizations of enrichment protocols to preferentially isolate T cells expressing high levels of CD34t, will further improve the anti-tumor reactivity of the resulting effector population, and the power of this methodology.

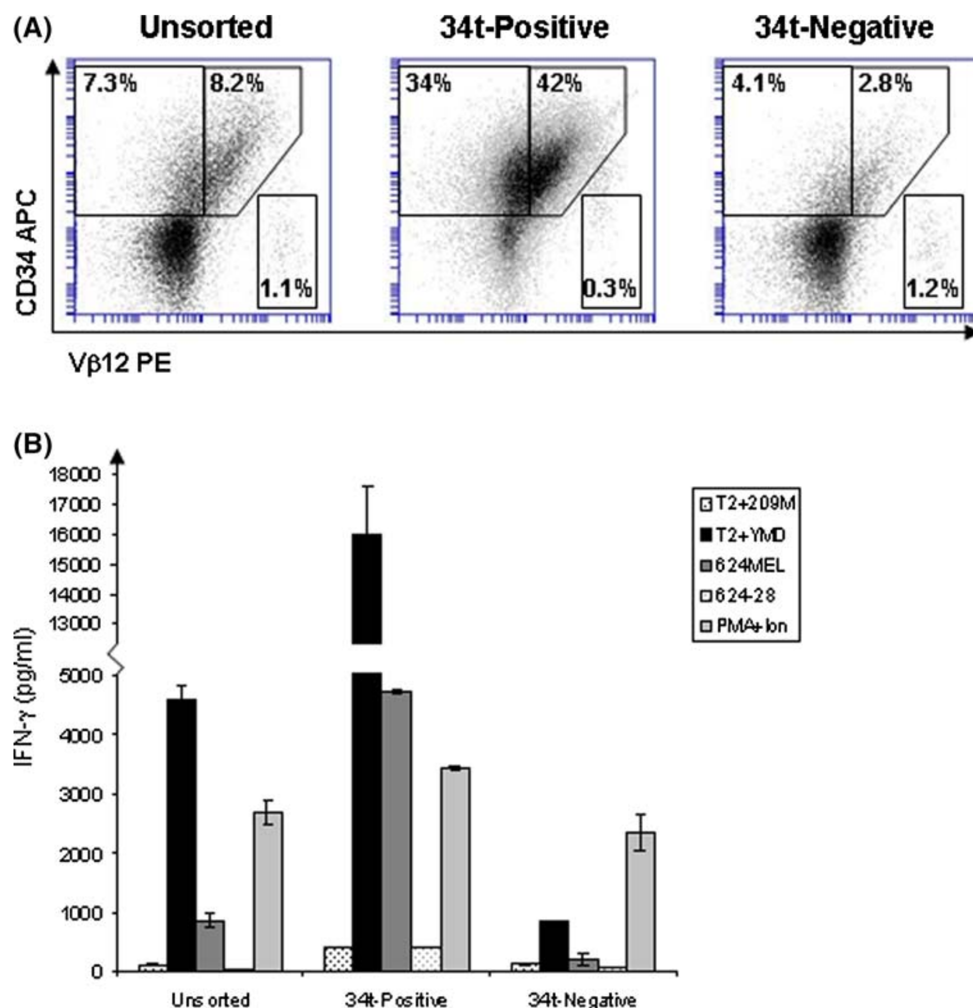

Fig. 3 Enrichment of redirected T cells using CD34-based stem cell isolation procedures results in dramatically enhanced antigen specific tumor targeting. T cells transduced with TIL 1383I TCR/CD34t retrovirus were separated in two fractions using Dynal CD34 progenitor cell selection system. After addition of anti-CD34 mAb-coated magnetic beads, unbound cells were collected as the "34t-Negative" fraction. The remaining cells were then dissociated from the beads and collected as the "34t-Positive" fraction. The "Unsorted" control fraction consisted of sham-sorted cells, exposed to the same buffers and procedures but not the anti-CD34 mAb-coated beads. **a** Cells from each of the three fractions were stained with mAbs specific for CD8, Vβ12 and CD34 or the respective isotype controls before exposed to 7AAD and analyzed by flow cytometry. All plots show the relative logarithmic immunofluorescence intensities of FL2 versus FL4 for the viable lymphocytes. **b** In parallel, 5×10^4 cells from each fraction were co-cultured overnight with 5×10^4 peptide-pulsed T2 cells, irrelevant gp100$_{209-217,210M}$ (209M) or cognate Tyrosinase$_{368-376}$ (YMD), or HLA-A2 positive melanoma 624 MEL cells or matched HLA-A2 negative 624-28 MEL cells. A mixture of PMA and ionomycin was used to induce polyclonal responses, serving as an internal positive control for each of the three T cell populations. The amount of human IFN-γ released into the co-culture supernatants was measured by sandwich ELISA and the respective concentrations of cytokine present in wells containing various combinations of effector and target cells were estimated. *Bars* represent the mean concentration and *error bars* the standard deviation between duplicate cultures. The data shown is from one representative experiment of three

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

The previous analysis used the TIL 1383I TCR, which can be detected using anti-Vβ12 mAbs. Another important advantage of the CD34t technology is that it enables enrichment for linked transgenes that cannot be directly monitored in live cells. Our HCV cl 3 TCR, which targets the HLA-A2 restricted 1406–1415 epitope from hepatitis C virus (HCV) NS3, is not bound by any available TCR-subfamily specific mAbs (data not shown). We transduced T cells with HCV cl 3 TCR/CD34t retrovirus and found that individual experiments had 8–42% CD34$^+$ T cells (data not shown). Using immunomagentic beads, dramatic enrichment in the percentage and functionality of transduced effectors was observed. A representative experiment is shown in Fig. 5. Compared to the unsorted population, this cell selection system led to greater than

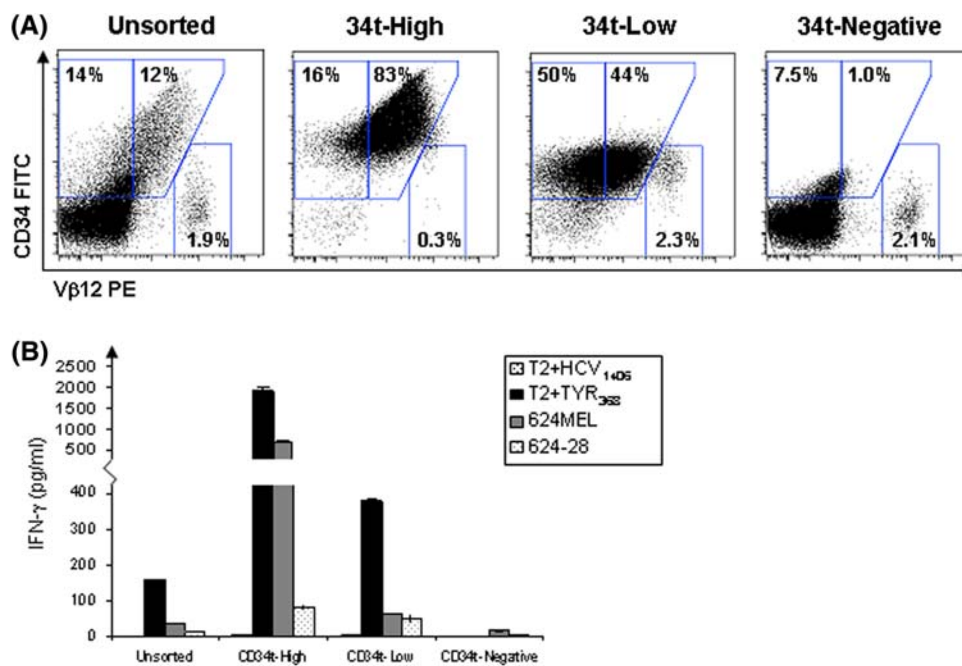

Fig. 4 Fluorescence-activated cell sorting of transduced T cells into fractions expressing distinct cell surface levels of CD34t. Activated PBL were transduced once with TIL 1383I TCR/CD34t retrovirus and expanded for 1 week. The beads were then removed and the T cells frozen. Upon thawing, cells were stained with anti-CD34 mAb and 7AAD and viable cells sorted by FACS into four different fractions. The CD34t$^+$ T cells were divided down the middle in two equal portions, "34t-High" (cells sorted as the half having highest CD34t expression) and "34t-Low" (the remaining CD34t$^+$ cells). In addition, CD34t$^-$ cells were isolated as the "34t-Negative" fraction. "Unsorted" cells were 7AAD$^-$ lymphocytes FACS isolated irrespective of their CD34t expression. **a** After overnight culture, cells from each of the FACS fractions were stained with mAbs specific for CD34, Vβ12 and CD8 before exposed to 7AAD and analyzed by flow cytometry. All plots show the relative logarithmic immunofluorescence intensities of FL2 versus FL1 for the viable lymphocytes. **b** In parallel, 5×10^4 cells from each fraction were co-cultured overnight with 2.5×10^4 peptide-pulsed, irrelevant HCV NS3$_{1406–1415}$ or cognate tyrosinase$_{368–376}$, T2 cells or 5×10^4 HLA-A2 positive melanoma 624 MEL cells or HLA-A2 negative 624-28 MEL cells. The supernatants were analyzed by sandwich ELISA and the concentrations of IFN-γ in wells containing the respective combination of effector and target cells were estimated. *Bars* represent the mean concentration and *error bars* the standard deviation between duplicate cultures. The data shown is from one representative experiment of two

sevenfold increase in the percentage of CD34t$^+$ cells in the positive fraction, and approximately 50% depletion of transduced cells in the negative fraction (Fig. 5a). There appears to be no selective bias for the enrichment or depletion of CD4$^+$ or CD8$^+$ T cells. The impact of CD34-based selection on T cell function was also analyzed (Fig. 5b). Upon co-culture with T2 cells pulsed with cognate peptide, effector cells from the positive fraction secreted about fourfold more IFN-γ than unsorted cells (Fig. 5b). Importantly, the anti-tumor reactivity against HCV NS3$^+_{1406–1415}$ HCC cells was enriched more than sixfold. As expected, the CD34 negative fraction had reduced reactivity compared to unsorted cells. All effectors were responsive to polyclonal stimuli and exhibited minimal reactivity versus non-cognate target cells. These results confirm that our CD34t expressing RV can be used to enrich for functional gene-modified cells regardless of our ability to monitor expression of the transgene of interest.

Discussion

Clinical responses upon TIL therapy were associated with shorter ex vivo culture [13] and longer telomere length of infused TIL that resulted in persistence [21, 22]. Although G418 selection enriches for gene-modified cells it concomitantly prolongs the ex vivo culture [23] and that may be detrimental to T cell survival in vivo and the clinical efficacy [13, 16]. Using the novel RV, ex vivo engineering, expansion, and enrichment of redirected T cells can be accomplished in a week, thus allowing quicker treatment with less cultured effector cells.

Many TCRs bind tetramer reagents or have an α- or β-chain that can be stained with available TCR-subfamily specific antibodies. Hence, TCR transduced T cells can often be labeled and enriched by indirect bead capturing or FACS. However, the CD34t-mediated enrichment is superior for several different reasons. Most importantly, direct binding of the TCR chains may trigger signaling

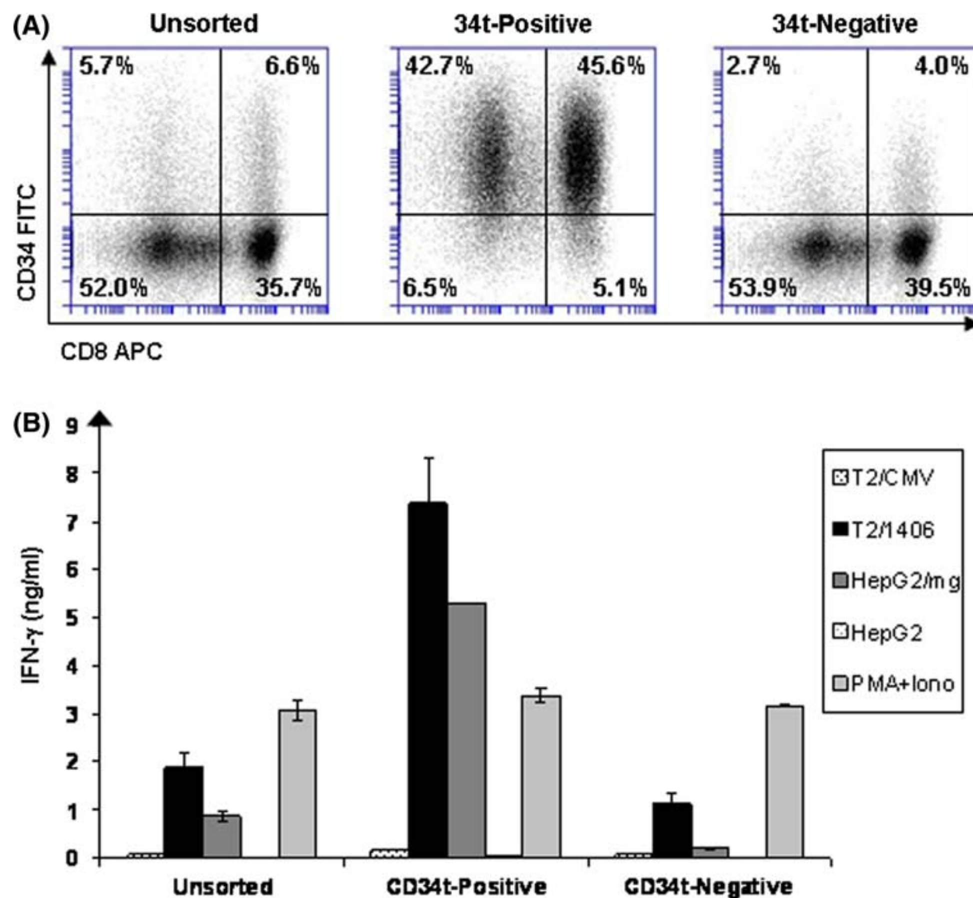

Fig. 5 CD34-based enrichment of the function of redirected T cells transduced with a non-traceable transgene. Activated PBL were transduced once with HCV cl 3 TCR/CD34t retrovirus, expanded for 1 week and separated in two fractions using the magnetic bead-based enrichment system. Cells bound to anti-CD34 mAb-coated magnetic beads were collected as the "34t-Positive" fraction, while the remaining cells constituted the "34t-Negative" fraction. The "Unsorted" control fraction consisted of sham-sorted cells. **a** Cells from each of the three fractions were stained with mAbs specific for CD34 (or its isotype control), CD8 and 7AAD. All plots show the relative logarithmic immunofluorescence intensities of FL4 versus FL1 for the viable T lymphocytes. **b** 1×10^5 cells from each fraction were co-cultured overnight with 1×10^5 target cells. Effectors were stimulated with T2 cells pulsed with irrelevant CMV_{pp65} peptide or cognate HCV $NS3_{1406–1415}$ peptide, or the HLA-A2 positive hepatocellular carcinoma cell line HepG2 with or without a HCV $NS3_{1406–1415}$ minigene. A mixture of PMA and ionomycin was used to induce polyclonal responses, which served as internal positive control for each of the sorted T cell populations. The concentrations of IFN-γ in the co-culture supernatants were estimated by sandwich ELISA. *Bars* represent the mean concentration and *error bars* the standard deviation between duplicate cultures. The data shown is from one representative experiment of five

events that negatively affect the future viability and reactivity of the effector cells [24]. Since CD34t lacks signaling domain, this marker will not trigger cellular activation upon engagement by antibodies. In addition, the technology developed for stem cell transplantation ensures that clinical grade reagents and apparatus to isolate $CD34^+$ T cells are readily available [25, 26]. Finally, when the expression of the transgene cannot be directly monitored in viable engineered cells the CD34t selection system provides a unique tool to enrich them.

The clinically available CD34-based selection systems have been exploited for enrichment of gene-modified T cells before [27], but only 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 donor lymphocyte infusions following allogeneic hematopoietic cell transplantation, as safety mechanisms against graft-versus-host disease [28, 29]. Regrettably, frequent development of $CD34^+$ GCV-resistant T cells jeopardizes the safety of suicide gene technology and has hampered the usefulness of this strategy [30].

When CD34 expression is used as a selectable marker for enrichment of TCR transduced T cells there is no selective pressure to generate cells positive for one

transgene but not the other. More importantly, even when such cells arise that will not constitute a major problem. The application of CD34 as an independent selectable marker rather than as part of a fusion protein also allows enrichment for a much wider range of transgenes, including cell surface receptors.

Since the endogenous and exogenous TCRs compete for expression, it is crucial to promote cell surface expression, pairing, and function of introduced TCRs [31]. Compared to codon optimization [32], addition of cysteines in the constant regions of TCRs [33, 34], introduction of mouse constant TCR regions [35] and modification of N-glycosylation sites [36] the CD34t-based selection technology is more generally applicable as any human TCR can be easily cloned into the RV developed through the use of a conserved *Stu*I restriction site in the constant region of human TCR β-chains (Fig. 1b). Notably, the CD34t selection system also allows for concomitant employment of other strategies and its combination with codon optimization, introduction of cysteines and modification of N-glycosylation sites are warranted in future studies.

The use of vectors co-delivering CD34t and TCRs also enable improved immune monitoring. Since CD34 is not expressed by untransduced 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 [37] 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, 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 govern localization, persistence, and function of adoptively transferred T cells.

Expression of CCR7 and/or CD62L divides human memory T cells into two functionally distinct subsets [38, 39]. 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 [3]. Expression of CD27 and CD28, markers of less differentiated T cells, on TIL was associated with anti-tumor activity in patients and novel protocols generating "young" TIL have recently been developed [40, 41]. In TCR-transgenic mice, naïve antigen specific T cells are readily available and upon appropriate activation, these may mediate potent anti-tumor activity [42, 43]. 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 technology will likely allow rapid engineering, selection, and infusion of quiescent redirected 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 [44]. 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, rapid CD34-based enrichment and infusion of resting T cells an attractive alternative. In addition, the risk of malignant transformation will likely be smaller when T cells rather than hematopoietic stem cells are transduced [45].

In conclusion, we provide proof of concept for a readily applicable enrichment technology that can be used to enrich genetically modified cells of almost any cell type expressing products that normally would be hard to select for. Thus, we assert that vectors employing the potent and clinically compatible CD34-based enrichment technology will not only improve feasibility and efficacy of adoptive transfer of gene-modified T cells, but will help make substantial advancements in cellular engineering in general.

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