

# Cancer Immunology Immunotherapy

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# Cancer Immunology Immunotherapy

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ISSN print edition 0340-7004  
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**Typesetters:** Scientific Publishing Services, Chennai, India

Printed on acid-free paper

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# In vitro generated anti-tumor T lymphocytes exhibit distinct subsets mimicking in vivo antigen-experienced cells

Shicheng Yang · Gattinoni Luca · Fang Liu ·  
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Received: 19 October 2010 / Accepted: 11 January 2011 / Published online: 9 February 2011  
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**Abstract** The T-lymphocyte pool can be subdivided into naïve (Tn), effector memory (Tem), and central memory (Tcm) T cells. In this study, we characterized in vitro short-term cultured anti-tumor human T lymphocytes generated by lentiviral transduction with an anti-tumor antigen TCR vector. Within 2 weeks of in vitro culture, the cultured T cells showed a Tcm-like phenotype illustrated by a high percentage of CD62L and CD45RO cells. When the cells were sorted into populations that were CD45RO+/CD62L-(Tem), CD45RO+/CD62L+(Tcm), or CD45RO<sup>low</sup>/CD62L+(Tn) and co-cultured with antigen-matched tumor lines, the magnitude of cytokine release from these populations for IFN $\gamma$  (Tn < Tcm < Tem) and IL-2 (Tn > Tcm > Tem) mimicked the types of immune cell responses observed in vivo. In comparing cell-mediated effector function, Tn were found to be deficient (relative to Tcm and Tem) in the ability to form conjugates with tumor cells and subsequent lytic activity. Moreover, analysis of the gene expression profiles of the in vitro cultured and sorted T-cell populations also demonstrated patterns consistent with their in vivo counterparts. When Tcm and Tem were tested for the ability to survive in vivo, Tcm displayed significantly increased engraftment and persistence in NOD/SCID/ $\gamma$ c<sup>-/-</sup> mice. In general, a large percentage of in vitro generated anti-tumor T lymphocytes

mimic a Tcm-like phenotype (based on phenotype, effector function, and increased persistence in vivo), which suggests that these Tcm-like cultured T cells may be optimal for adoptive immunotherapy.

**Keywords** Gene therapy · Lentiviral vector · T-cell receptor · Central memory cells · Effector memory cells · Tumor immunity

## Introduction

Adoptive cell therapy (ACT) using tumor-infiltrating lymphocytes (TIL) is a potent treatment for patients with metastatic melanoma and can mediate objective responses in 50–70% of patients [1–3]. However, not all patients have pre-existing tumor reactive T lymphocytes, and these tumor reactive lymphocytes are generally only found in melanoma patients, which limits the broad application of this approach. The transduction of peripheral blood lymphocytes (PBL) with genes encoding anti-tumor TCRs using  $\gamma$ -retroviruses readily renders autologous PBL from any patient into tumor killing T lymphocytes in vitro [4]. Administration of anti-MART-1 TCR-engineered PBL in patients with advanced metastatic melanoma resulted in a 30% objective response rate [5, 6] and holds the promise for the treatment of other types of cancer. Genetic modification of T cells in vitro with anti-tumor TCRs, followed by robust in vitro expansion, may avoid immune tolerance observed in vivo and can generate sufficient numbers of specific anti-tumor T cells for patient treatment.

For TCR gene therapy, current protocols using  $\gamma$ -retroviral vectors require T cells to be actively dividing for efficient gene integration into cellular DNA. The need for fully dividing T cells limits the number of cells that can be

**Electronic supplementary material** The online version of this article (doi:10.1007/s00262-011-0977-7) contains supplementary material, which is available to authorized users.

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transduced and often requires a second rapid expansion (REP) in order to generate enough cells for clinical application. However, this second expansion causes cells to become fully differentiated and exhibit an effector memory phenotype that may impede *in vivo* persistence [7] and *in vivo* tumor killing efficacy [8]. Compared with  $\gamma$ -retroviral vectors, lentiviral vectors efficiently transduce non-dividing cells. The minimal requirement for lentiviral vector-mediated transduction of quiescent T cells is that T cells enter into the G1 phase of the cell cycle. Following anti-CD3 activation, quiescent T cells easily move into the G1 phase within hours [9, 10]. The use of anti-CD3/CD28 beads provided a simple activation method for lentiviral vector-mediated transduction and transgene expression [9, 11], and a clinical-scale transduction protocol has been reported to yield  $>1 \times 10^{10}$  transduced T cells in a minimal culture period [9].

In humans, the T-lymphocyte pool can be subdivided into naïve antigen-inexperienced cells (Tn) and following antigen encounter into effector memory (Tem) and central memory (Tcm) T cells [12–15]. Tcm cells are thought to contain a long-lived memory cell population displaying a capacity for self-renewal associated with high levels of phosphorylated transcription factors [16]. Recent studies suggest that there are additional subsets of CD8<sup>+</sup> T-memory cells in mice [17] and in humans [18]. Central memory CD8<sup>+</sup> T cells and effector memory CD8<sup>+</sup> T cells have been identified in humans and animals and can be distinguished in part by the expression level of CCR7, CD62L [19] and the secretion of cytokines [15]. Animal studies suggest that central memory T cells not only survive longer after ACT [7] but also confer superior anti-tumor reactivity compared with effector memory T cells and can lead to the eradication of large established tumors [20].

The generation of an active population of memory T cells is pivotal for effective vaccine and cell-based therapies to fight infectious diseases and cancer [21]. The phenotypic and functional characterization of immune T cells after antigen challenge *in vivo* has been well studied [15, 22]. However, the immune-related characteristics of *in vitro* generated anti-tumor antigen TCR-engineered T cells remain to be elucidated. In this report, we systematically analyzed *in vitro* TCR gene-engineered T lymphocytes for their phenotype, function, and *in vivo* engraftment to determine the optimal type of cell for use in adoptive cell therapy.

## Materials and methods

### Cell culture

PBL used in this study were obtained from healthy donors or metastatic melanoma patients seeking

treatments at the Surgery Branch, National Cancer Institute, under approved clinical protocols. Briefly, PBL were collected by leukapheresis, and lymphocytes were separated by Ficoll/Hypaque cushion centrifugation, washed in HBSS, and resuspended at a concentration of  $1 \times 10^6$ /ml in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 300 IU/ml IL-2 and 5% heat-inactivated human AB serum (Valley Biomedical, Winchester, VA). Melanoma cell lines included MART-1-positive HLA-A2<sup>+</sup> 526 and MART-1-positive HLA-A2<sup>−</sup> 938. 293T cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen). All cell lines were cultured at 37°C in a 5% CO<sub>2</sub> humidified incubator.

### Vector construction and lentivirus preparation

The lentiviral constructs utilized were derived from pRRLSIN.cPPT.MSCV/GFP.wPRE harboring a green fluorescent protein (GFP) gene driven by the murine stem cell virus (MSCV) U3 promoter [23]. Woodchuck hepatitis virus response element (wPRE) was replaced with the truncated form oPRE, where the residual X protein and its promoter were deleted [24]. The oPRE sequence was synthesized and fused with Sal I and EcoR I restrictive enzyme sites (Epoch Biolabs, Missouri City, TX) and cloned into corresponding sites of pRRLSIN.cPPT.MSCV/GFP.wPRE to yield vector pLLV.GFP.oPRE. A lentiviral vector expressing the gp100 TCR alpha and beta chains of DMF5 TCR targeting melanoma antigen MART-1 [25] was previously described [9, 26, 27]. All constructs are confirmed by restrictive enzyme digestion and sequencing.

For lentivirus preparation, the day before transfection,  $20 \times 10^6$  293 T cells were plated onto 150-mm<sup>2</sup> poly-D-Lysine-coated plates (BD Biosciences, San Jose, CA) using 15 ml of culture medium. On the day of transfection, the medium was replaced with 15 ml fresh medium 3 h before transfection. Each plate received plasmid DNA 55  $\mu$ g (transfer vector 22.5  $\mu$ g, VSV-G 7.5  $\mu$ g, pMDLg/pRRE 15  $\mu$ g and pRSV-Rev 10  $\mu$ g) and 165  $\mu$ l lipofectamine 2000 (Invitrogen). Before transfection, 10 ml of culture medium was removed from the culture dishes and complexes of plasmid DNA and Lipofectamine 2000 were added evenly onto the medium. Six hours after transfection, the plates were washed twice with PBS and 20 ml of fresh medium was added. The supernatant was collected 48 h post-transfection, and cell debris was removed by centrifugation at  $2,000 \times g$  for 10 min. Supernatant containing viruses was tittered using a p24 kit (ZeptoMetrix, Buffalo, NY) and was either used directly or stored at  $-80^\circ\text{C}$ .

### The generation of anti-tumor T lymphocytes and FACS analysis/sorting

For cell preparation, briefly PBL were activated using anti-CD3/CD28 beads overnight, and next day, the cells were transduced with lentiviral vector harboring anti-tumor TCR by spinoculation as previously described in detail [9]. Six hours post-transduction, the cells were transferred to 75-cm<sup>2</sup> flask and maintained below concentration of 10<sup>6</sup>/ml. Cell surface expression of CD3, CD4, CD8, CD27, CD127, CCR7, CD28, CD70, CD95, CD137, CD57, CD62L, and CD45RO was measured using fluorescein isothiocyanate (FITC)-, allophycocyanin (APC)-, phycoerythrin (PE) PE-CY-7-, or APC-CY-7- conjugated antibodies (BD Biosciences, San Jose, CA). MART-1:27-35 tetramer (PE or APC) was used (iTag MHC Tetramer, Beckman Coulter, Fullerton, CA) to verify TCR gene transfer. Immunofluorescence staining was analyzed as the relative log fluorescence of live cells, determined using a FACScan flow cytometer (BD). A combination of forward angle light scatter and propidium iodide staining was used to gate out the dead cells, and 1 × 10<sup>5</sup> cells were analyzed. All FACS data were analyzed using FlowJo 8.1.1 software (Tree Star Inc., Ashland, OR). For cell sorting, 14-day cultured cells were washed twice with PBS and stained with anti-human CD8- or CD3-PE-Cy-7, CD45RO-FITC, CD62L-APC (BD Biosciences), and propidium iodide (PI) (50 µg/ml). Before sorting, the stained cells were passed through 40-µm nylon cell strainer (BD). The cells were either gated on CD8 or gated on CD3 subsets depending on the specific experiment and using differentiation markers CD45RO and CD62L to yield CD62L-/CD45RO+ (Tem), CD62L+/CD45RO+ (Tcm), and CD62L+/CD45RO- (Tn) populations. The sorting was performed on a FACSARIA I cytometer (BD) equipped with 2 laser system (488-nm blue laser and 633-nm red laser); the cells were passed through 70-µm nozzle under pressure of 70 psi. The cytometer was driven by BD FACSDiva version 6.1.3 software.

### Illumina gene expression array

Total RNA from sorted Tem, Tcm, and Tn CD8 subpopulations was extracted, and 200 ng of total RNA was used to generate biotin-labeled cDNA using Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX). Biotin-labeled cDNA (750 ng) was hybridized to Sentrix BeadChip Array for Gene Expression (Human Ref-12 V2, Illumina, San Diego, CA) and incubated at 58°C for 16–20 h in an Illumina hybridization oven with rocker speed at 5. Beadchips were washed and stained according to Illumina's protocol. Arrays were scanned by Illumina chip scanner, and images were analyzed by Bead Studio

(Illumina, San Diego, CA). Data were exported and processed using Genespring GX 11 (Agilent, Santa Clara, CA). Differentially regulated genes between different subpopulations were identified by statistical significance  $P < 0.05$  and fold change  $> 2$ . Significantly regulated genes were subjected to further analysis using Ingenuity Pathway software (Ingenuity, Redwood City, CA).

### Effector–target conjugate formation assay

The assay for effector–target cell conjugate formation has been previously reported [28]. Briefly, melanoma line 526 was labeled with CFSE at a concentration of 1 µM using CFSE cell proliferation kit (Invitrogen) according to product manual; 1 × 10<sup>7</sup> melanoma 526 cells were labeled, and the cells were cultured for 48 h prior to use. Lentiviral TCR-vector-transduced PBL (14 days in culture) were sorted as described earlier. The co-culture was initiated by combining 3 × 10<sup>5</sup> each of target and effector cell in 14-ml polypropylene round-bottom tubes (BD) by centrifugation at 137×*g* for 10 s. At the end of incubation, the cells were stained with CD3 PerCP, CD8 APC, and MART-1 PE tetramer to analyze for T cell:tumor cell conjugates by FACS. FACS data were analyzed using FlowJo software. The percentage of effector–target conjugate formation was determined using double positive cells divided by CFSE-labeled tumor cells; the statistic analysis was based on mean ± SD from triplicate samples.

### Measurement of lymphocyte reactivity to antigen

Transduced PBL effector cells (1 × 10<sup>5</sup>) were co-cultured with melanoma lines (1 × 10<sup>5</sup>) in a final volume of 0.2 ml in each well of a round-bottom 96-well plate. Cell culture supernatants were harvested and assayed 16 h later for IFN $\gamma$  and IL2 by ELISA kit (Pierce Endogen, Rockford, IL). The culture supernatants were diluted to be in the linear range of the assay. Results represent mean ± SD of triplicate cultures. The ability of transduced PBL to lyse melanoma tumor lines was evaluated using a <sup>51</sup>Cr assay as described [29]. Briefly, 10<sup>6</sup> tumor cells were labeled for 1 h at 37°C with 100 µCi of <sup>51</sup>Cr (GE Healthcare, Piscataway, NJ). Labeled target cells (2 × 10<sup>3</sup>) were co-cultured with effector cells at the ratios indicated for 4 h at 37°C in 0.15 ml of medium. Harvested supernatants were counted using a MicorBeta TriLux instrument (Perkin Elmer, Waltham, MA). Total and spontaneous <sup>51</sup>Cr release was determined by incubating 2 × 10<sup>3</sup> labeled target cells in either 2% SDS or medium alone for the aforementioned conditions, respectively. Each data point was determined as a mean of quadruplicate wells. The percentage of specific lysis was calculated as indicated in figure legend.

# Engraftment of Tem and Tcm in NOD/SCID/ $\gamma$ c<sup>-/-</sup> mouse model

Sorted CD3 Tem and Tcm subpopulations were counted and suspended in PBS, and  $1.7 \times 10^6$  cells were injected intravenously into NOD/SCID/ $\gamma$ c<sup>-/-</sup> mice without supplement of IL2. Thirty-seven days later, mice were killed and T lymphocytes were isolated from spleen, lymph nodes (LN), and lung. T cells from lymph node (LN) spleen were lysed with ACK lysing buffer (BioWhittaker) followed by two PBS washes. T cells from lung were minced and tissue debris was removed. Lymphocyte separation medium (MP Biomedicals) was used to purify T cells by centrifugation

at  $2,000 \times g$  for 20 min. After collecting lymphocytes, and washing twice, the cells were quantitated and subject to FACS analysis.

## Results

### Phenotype and activity of in vitro generated anti-tumor T lymphocytes

Using overnight activation with anti-CD3/CD28 beads followed by lentiviral vector-mediated transduction (Fig. 1a), engineered cells from three donors were

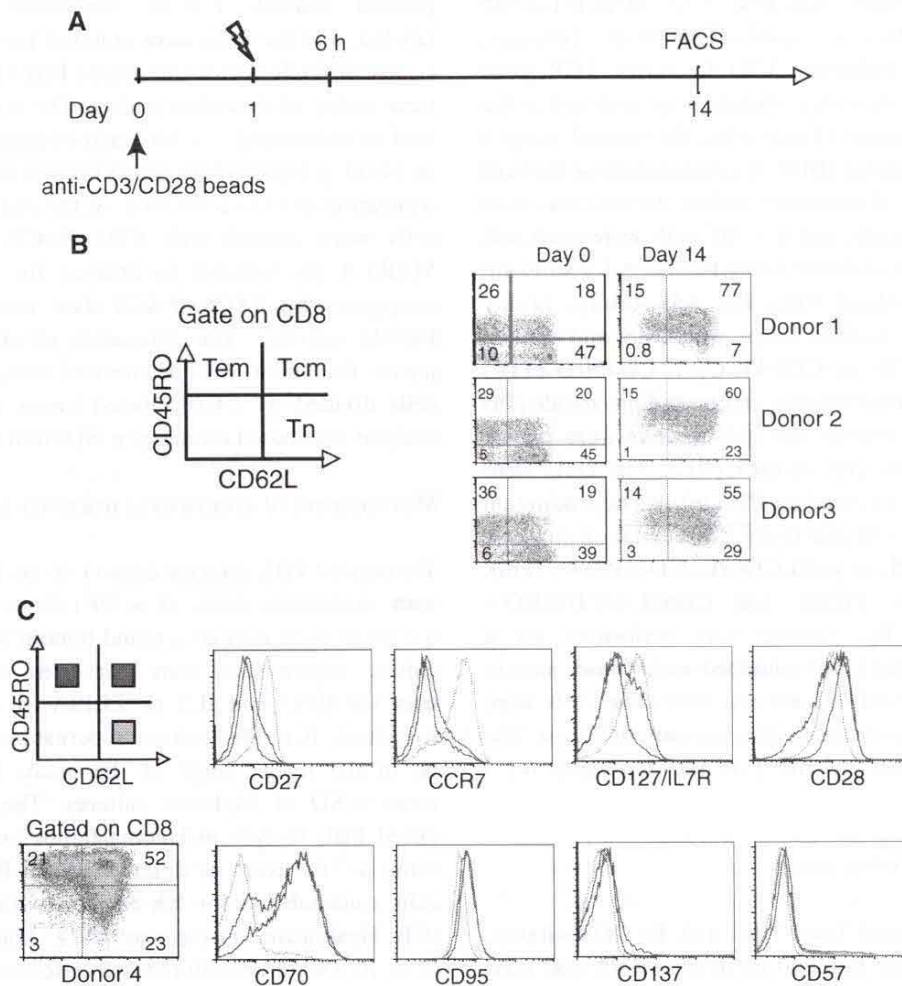


**Fig. 1** The in vitro cultured anti-tumor T lymphocytes exhibit distinct populations defined by differentiated markers CD45RO and CD62L. **a** Schematic illustration of in vitro generated anti-tumor T lymphocytes. PBMC were activated by anti-CD3/CD28 beads on day 0, and on day 1 post-stimulation, the cells were transduced with lentiviral vector harboring MART-1 antigen TCR and maintained in culture for 14 days. **b** FACS analysis of in vitro cultured T lymphocytes defined by CD45RO and CD62L. The in vitro cultured T lymphocytes were gated on CD8 T cells and analyzed using differentiated markers CD45RO and CD62L. The phenotype of the

cultured T cells from 3 donors at day 0 and day 14 is shown in dot plots. **c** The in vitro cultured anti-tumor T lymphocytes exhibit distinct populations defined by differentiated markers CD45RO and CD62L. Cells were gated on CD8, analyzed for CD45RO and CD62L expression followed by the individual markers as indicated. The expression of individual markers CD27, CCR7, etc. in each quadrant on left was overlaid on the right. The histograms from red, blue, and green indicate cells from Tem, Tcm, and Tn, respectively. Tem, effector memory T cells; Tcm, central memory T cells; Tn, naïve T cells. PBMC derived from donor 3 (color figure online)

expanded and harvested at day 14, and the phenotype of CD8<sup>+</sup> T cells was analyzed using differentiation markers CD45RO and CD62L. As shown in Fig. 1b, the Tcm cells were the least abundant at day 0, while this phenotype was the most plentiful at day 14 following transduction and in vitro culture. The phenotype of the three distinct subpopulations was further analyzed (Fig. 1c). The differentiation markers CD27, CCR7, and CD127/IL7R were preferentially expressed on the Tn population, while the differentiation marker CD70 showed the opposite pattern, i.e., the lowest expression occurred on the Tn population. We did not observe significant differences in other markers analyzed (CD95, CD137, CD28, and CD57).

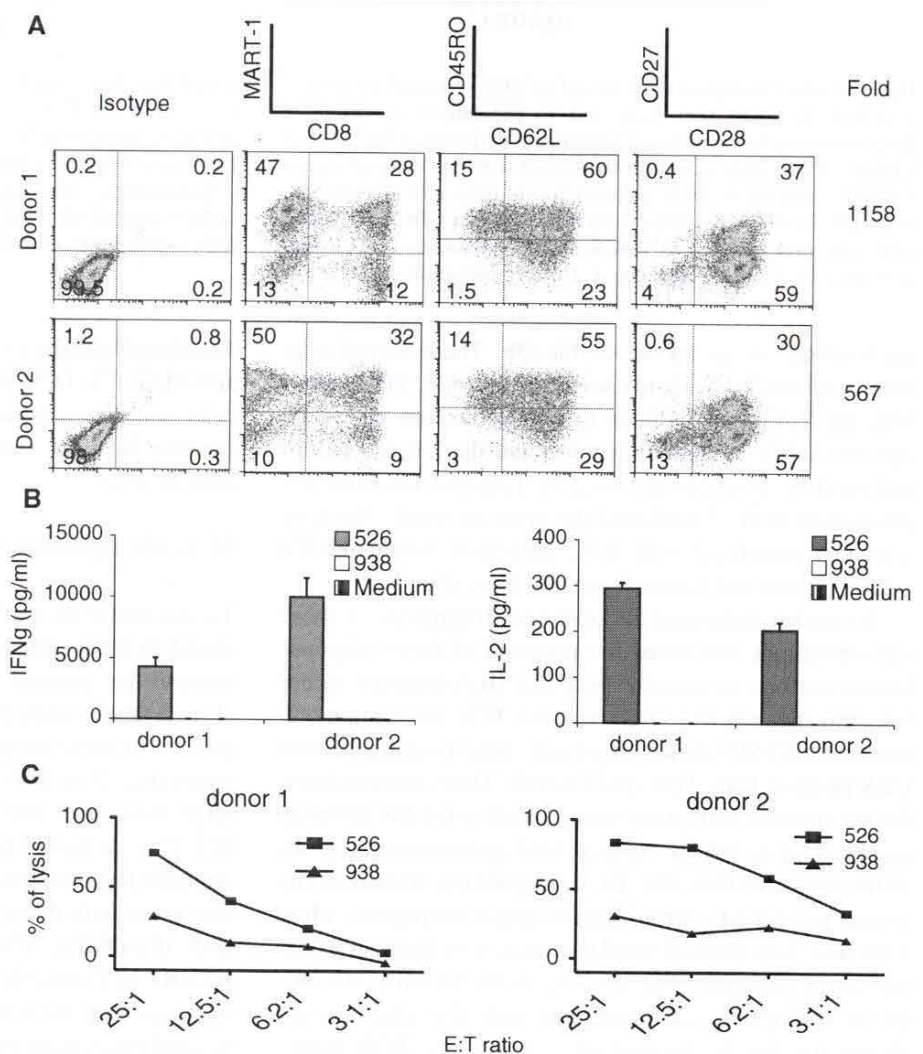
To verify the functional activity of these gene-engineered cells, PBL from two donors were transduced and expanded for 14 days and the TCR expression and the phenotype of T cells were analyzed using MART-1 tetramer and differentiation markers (Fig. 2a). The fold expansion within 14 days was illustrated on right. The specific TCR used for transduction is restricted for HLA-

A0201 and renders these cells reactive against HLA-A2-expressing tumors (i.e., melanoma line 526). The transduced cells demonstrated effector functions including cytokine secretion (IFN $\gamma$  and IL-2, Fig. 2b) and specific tumor cell lysis (Fig. 2c). To determine the reproducibility of this genetic modification protocol, we tested a total of six donors in three independent experiments (Supple Table 1). In summary, the PBLs from melanoma patients could be efficiently transduced with an anti-tumor TCR ( $70.6 \pm 18.4$ ), robustly expanded ( $568.7 \pm 355.8$ ), and the majority of these T cells showed Tcm-like phenotype.

#### Function of in vitro cultured TCR gene-engineered T lymphocytes

To determine the functional activity of the phenotypically distinct T-cell subpopulations, CD8<sup>+</sup> Tem, Tcm, and Tn (defined by CD45RO and CD62L) cells were sorted for the analysis of effector function (Fig. 3a). The purity of sorted cells and the expression of anti-tumor TCR MART-1 on

**Fig. 2** Biological activity of the in vitro cultured anti-tumor T lymphocytes. **a** Phenotypical analysis of transduced PBMC at day 14. Cells were gated for CD3 and further analyzed using a panel of differentiation markers as denoted on top of each FACS image. The fold expansion within 14 days was shown on right of each donor. **b** Cytokine induction. Transduced PBMC were co-cultured with melanoma lines, and cytokine production of IFN $\gamma$  and IL-2 was measured by ELISA. **c** Specific lysis of melanoma lines. The specific lytic activities of transduced CD8<sup>+</sup> T cells were determined by co-culture with <sup>51</sup>Cr-labeled tumor cells at the indicated ratio and the release of <sup>51</sup>Cr was measured. The percent cell lysis was calculated using the formula [(specific release – spontaneous release)/(total release – spontaneous release)]  $\times$  100. Results representing mean of triplicate cultures were plotted. Both melanoma lines express MART-1, 526 is HLA-A2 positive, and 938 is HLA-A2 negative



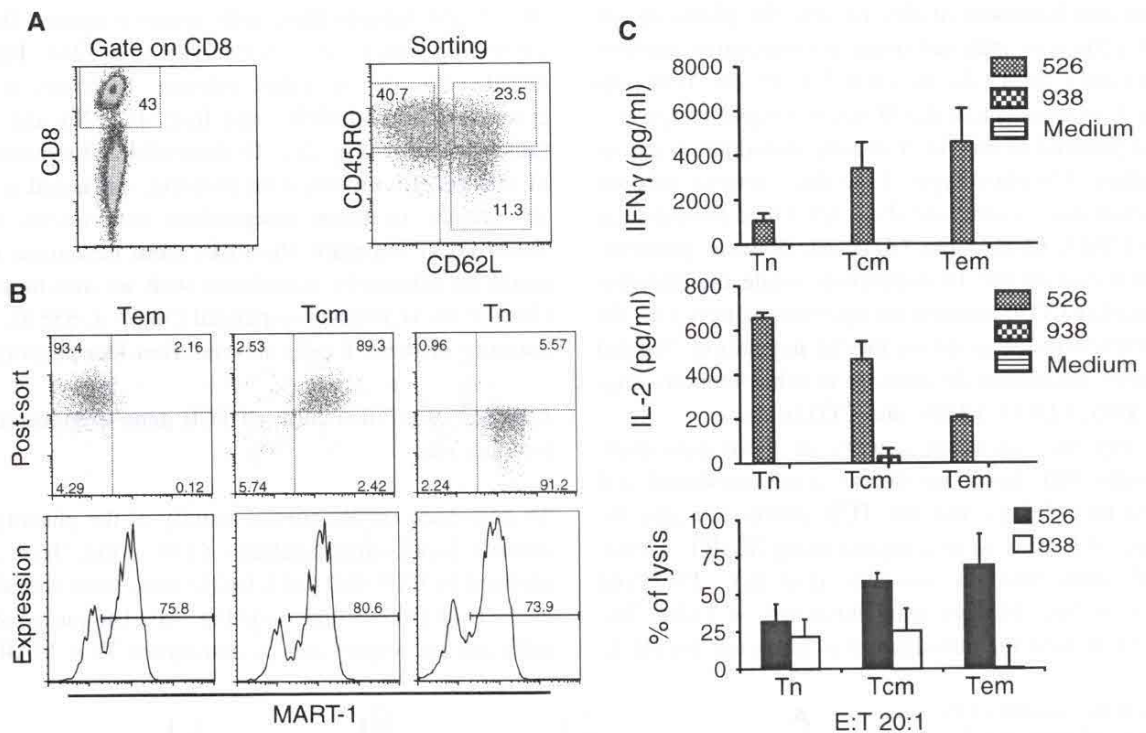


**Fig. 3** Distinct biological activities of in vitro generated anti-tumor T cells from sorted Tem, Tcm, and Tn populations. **a** Sorting of differentiated subpopulations of CD8 T cells defined by CD45RO and CD62L. PBMC were activated, transduced with the lentiviral vector harboring anti-tumor TCR, cultured for 14 days post-transduction, and CD8 T cells were sorted using CD45RO and CD62L. **b** Sorted cells were post-analyzed by FACS, and the expression of TCR was evaluated by Tetramer staining. **c** Distinct biological activities of

sorted Tem, Tcm, and Tn subpopulations. The sorted subpopulations representing Tn, Tcm, and Tem were co-cultured with tumor lines, and the secretion of IFN $\gamma$  and IL2 was determined by ELISA (data are the mean of triplicate cultures). To measure the lytic activity of sorted subpopulations, cells were co-cultured with  $^{51}\text{Cr}$ -labeled tumor cells at the indicated ratio and the release of  $^{51}\text{Cr}$  was measured. Results representing mean of triplicate cultures were plotted

each subset are as shown in Fig. 3b. These sorted cells when co-cultured with melanoma lines (526, HLA-A2+; 938, HLA-A2-) are shown to exhibit distinct biological activities (Fig. 3c). Tem demonstrated the greatest potential for IFN $\gamma$  synthesis followed by Tcm and Tn, while the production of IL-2 displayed the opposite trend. The lytic activities correlated with IFN $\gamma$  induction, where the Tn subpopulation had minimal specific lysis (Fig. 3c).

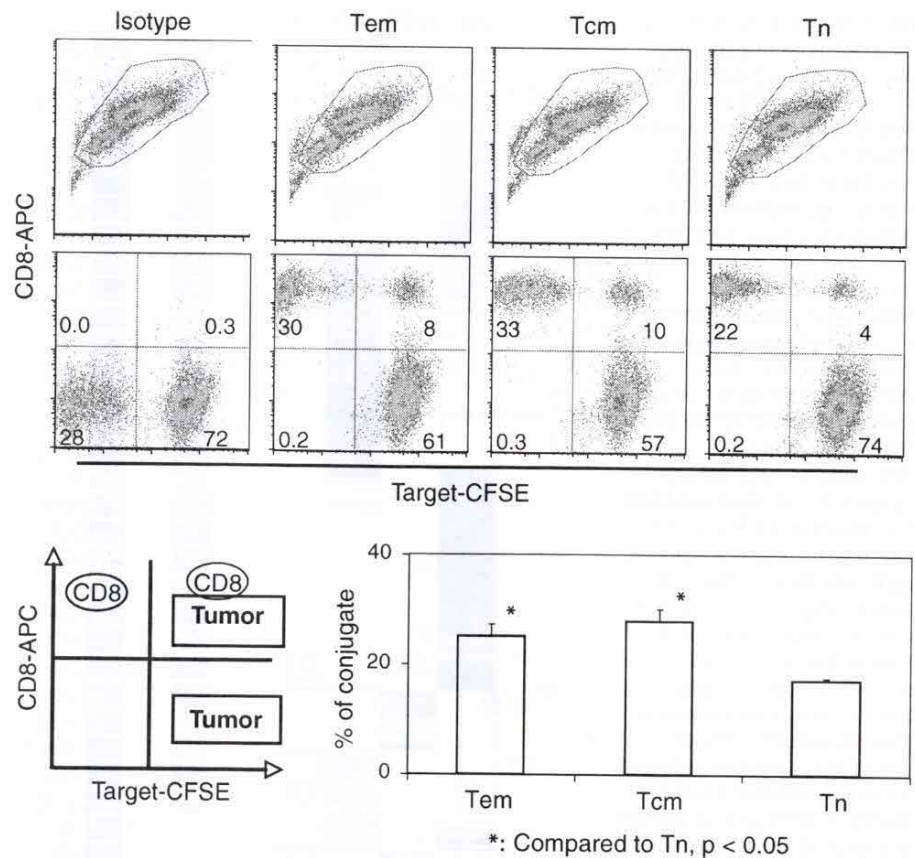
To further determine the intrinsic differences of these subpopulations, we tested the potential of these subpopulations to form conjugates with the HLA-matched tumor line 526 (the positive target cell for TCR recognition). In this assay, CFSE-labeled target cells were co-cultured with CD8-positive Tem, Tcm, and Tn cells. Thirty minutes later, the co-cultured cells were stained with anti-CD8 antibody and subject to FACS analysis and quantitation (Fig. 4). After normalization, the Tn subpopulation displayed the lowest potential to form effector–target conjugates, while Tem and Tcm showed similar activities in forming effector–target conjugate (Fig. 4). The ability to form effector–target conjugates was consistent with the lytic activity shown in Fig. 3. Interestingly, while the TCR gene-

transduced and the ex vivo cultured Tn cells expressed the anti-MART-1 TCR at equal levels to the Tem and Tcm cells, they demonstrated the lowest potential for effector function in vitro, similar to the biological activity of Tn cells in vivo.

#### Molecular signatures of in vitro cultured T lymphocytes

To determine the molecular signature of TCR gene-transduced in vitro cultured T cells, we performed microarray analysis on purified Tem, Tcm, and Tn subpopulations (Fig. 5 and supplementary Table 2). Gene expression profiles of these subpopulations exhibited dramatic differences (Fig. 5 on left). We amplified specific regions of the large heat map for detailed presentation on the right (C1–C5). In the C1 region, we focused on the genes more abundantly expressed in Tem followed by Tcm and Tn, and consistent with other reports [16], we observed up-regulation of EOMES, TNF, ID2, IFNG, GZMB, GZMH, and KLRB1 in Tem cells. In the C2 region, we amplified a list of genes that were highly expressed in Tcm population. Several genes were over-expressed in Tcm including IRX3,

**Fig. 4** Tn population shows reduced potential for effector–target formation. The sorted TCR-engineered CD8 subpopulations defined by CD45RO and CD62L were co-cultured with CFSE pre-labeled tumor line 526. At the end of incubation, the cells were stained with CD8 and T cell:tumor cell conjugate visualized by FACS analysis. The lower left panel is a schematic illustration showing the potential interactions of co-cultured cells. The effector–conjugate formation was calculated using conjugated population divided by un-conjugated in each subpopulation and presented as percentage as shown in the figure on the bottom right. The data represent the mean  $\pm$  SD from triplicate co-culture. The percentage of conjugate formation from Tem, Tcm versus Tn showed statistically significant,  $P < 0.05$



GATA3, ITGB1, TNFRSF4, and LILRB3 but the significance of these results remains to be elucidated. In C3, C4, and C5, we showed 3 regions with genes highly expressed on Tn subpopulation and observed several genes previously reported to be associated with Tn cells (CD127/IL7R, STAT1, LEF1, and CCR7). The molecular signature markers CD27, CCR7, CD127/IL7R, and CD70 (Fig. 2) identified by FACS analysis were confirmed by microarray analysis (Fig. 5).

#### Tcm show enhanced engraftment and persistence in NOD/SCID/ $\gamma$ c<sup>-/-</sup> mice

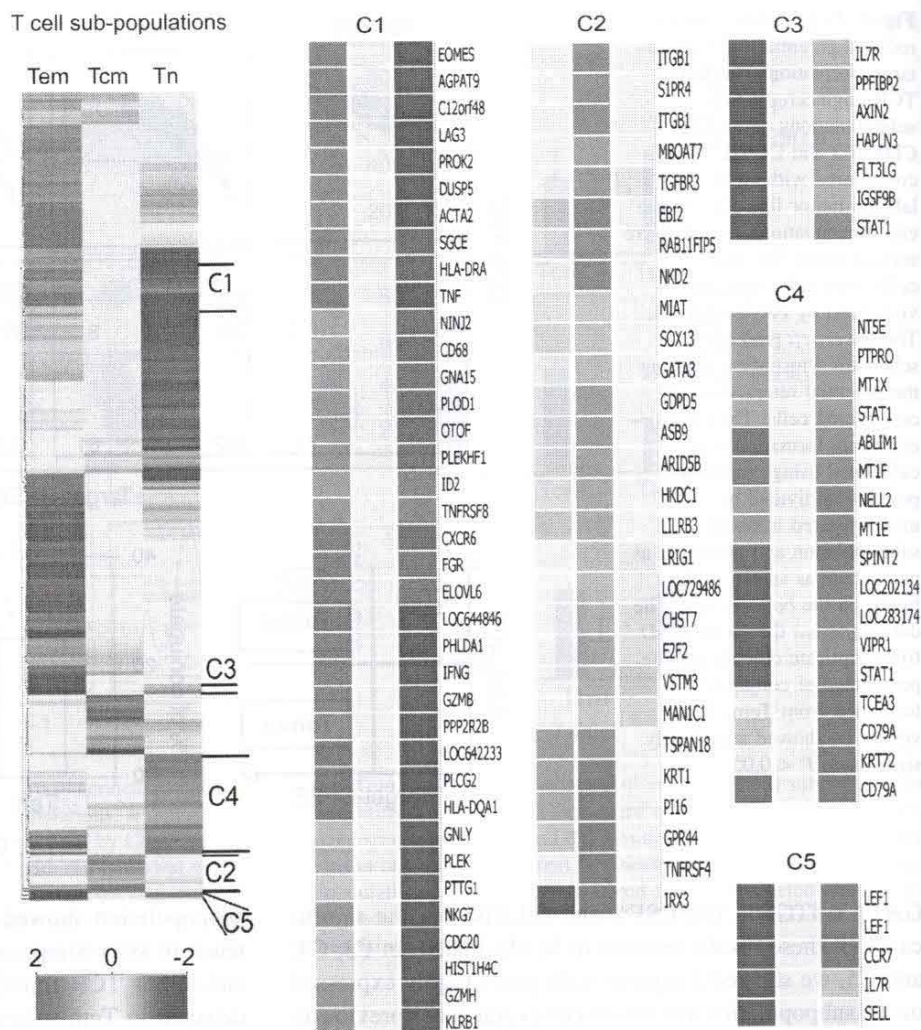
There is a growing evidence that less-differentiated T lymphocytes are more effective in vivo in terms of persistence and tumor treatment [7, 8, 20, 30]. To compare the in vivo engraftment of human T cells in an immunodeficient mouse model, we transduced PBL and expanded these cells for 2 weeks, followed by cell sorting into Tem and Tcm populations defined by CD45RO and CD62L (supplementary Fig. 1, the number of Tn cells was insufficient for in vivo studies). Tem and Tcm were injected into NOD/SCID/ $\gamma$ c<sup>-/-</sup> mice ( $1.7 \times 10^6$  cells per mouse) without any exogenous cytokine support. Thirty-seven days post-injection, the mice were killed and lymphocytes from spleen, LN, and lung were extracted. The Tcm

subpopulation showed significant engraftment and persistence in vivo compared with the Tem subpopulation. The anti-tumor TCR transduced by lentiviral vector was also detected in Tcm subpopulation (Fig. 6). The total number of infused human cells in spleen, LN, and lung was calculated and the data indicated that the Tcm had a significantly increased engraftment and persistence compared with Tem in the NOD/SCID/ $\gamma$ c<sup>-/-</sup> mouse model (Fig. 6).

#### Discussion

When T lymphocytes encounter foreign antigen, they undergo robust expansion in a short period enabling sufficient numbers of antigen-specific T cells to eradicate foreign antigens. The outcome of this immune response usually generates two types of memory T cells, Tem and Tcm, which co-exist with the antigen-inexperienced Tn populations [12–15]. Tcm and Tem cells can be distinguished phenotypically and functionally. Compared with Tem, Tcm cells express L-Selectin/CD62L and chemokine receptor CCR7 and secrete IL-2 rather than IL-4 and IFN $\gamma$ . The expression of CD62L is also characteristic of Tem and Tn cells' ability for cell extravasation through high endothelial venules and migration to areas of secondary lymphoid organs [12, 13], whereas Tem cells lack CD62L

**Fig. 5** Sorted Tem, Tcm, and Tn populations display unique gene expression patterns. The in vitro cultured CD8<sup>+</sup> T lymphocytes, further defined by CD45RO and CD62L, were sorted into Tcm, Tem, and Tn populations, and total RNA was extracted for analysis of gene expression profiles by cDNA microarray. Two-way hierarchical clustering of sorted T-cell samples was conducted and 2-fold differentially expressed genes were chosen for analysis. A set of 597 probe IDs was identified as differentially expressed among cultured T-cell subsets and used for clustering. Each row in the heat map represents a gene, and each column represents a T subset sample. As shown in the color bar, red indicates higher than median expression (up-regulation) and blue indicates lower than median expression (down-regulation). The five main gene clusters are indicated on the right from C1 to C5. Higher magnification of clusters is displayed on the right. Gene symbols are shown next to the heat map (color figure online)



and are resident in tissues such as lung, liver, and gut [31]. Antigen-specific memory T cells against viruses or other microbes can be found in both Tcm and Tem subsets. Tcm cells have been shown to confer superior protection against viruses [22], bacteria [22], and cancer [20] in several different model systems compared with Tem cells. Recently, a distinct subset of self-renewing human memory CD8<sup>+</sup> T cells were isolated from both Tcm and Tem cells [18].

ACT using autologous T lymphocytes genetically modified to express anti-tumor TCRs can mediate tumor regression, and this strategy can now be applied to patients with common epithelial cancers [3, 32, 33]. At present, what is not well understood is how in vitro culture and gene transduction affect the T cells' phenotype and function. Interestingly, when we analyzed the genetically modified and in vitro expanded T lymphocytes defined by differentiated markers CD45RO and CD62L, we always observed the same distribution of T-cell subsets as are observed in vivo cells, i.e., Tem (CD45RO+CD62L<sup>-</sup>), Tcm (CD45RO+CD62L<sup>+</sup>), and Tn (CD45RO-CD62L<sup>+</sup>). The

percentage of cells in each subpopulation varied with extent of in vitro culture, but usually at day 14 the Tcm dominated the populations. In this report, we demonstrate that the majority of these TCR gene-engineered, in vitro cultured T cells not only have the phenotype of Tcm cells but also mimic the biological properties of in vivo Tcm cells. A similar phenotypic distribution has been reported using  $\gamma$ -retroviral vector transduction of a chimeric antigen receptor in human T cells [34].

In vitro generated Tcm cells have comparable cytokine and lytic abilities as in vitro generated Tem cells, but their survival in NOD/SCID/ $\gamma$ c<sup>-/-</sup> mice is significantly enhanced compared with Tem cells. The gene expression pattern by microarray analysis of cultured Tem, Tcm, and Tn of CD8 T cells clearly indicated that these three subpopulations of cells were at different differentiation stages (Fig. 5). These data are consistent with gene expression patterns known to be higher at Tem including EOMES [35], TNF [36], ID2 [37], IFNG [38], GZMB [39], GZMH [40], and GNLY [41] and to be lower at Tn including IL7R [42], STAT1 [43], LEF1 [44], and CCR7 [45]. We also

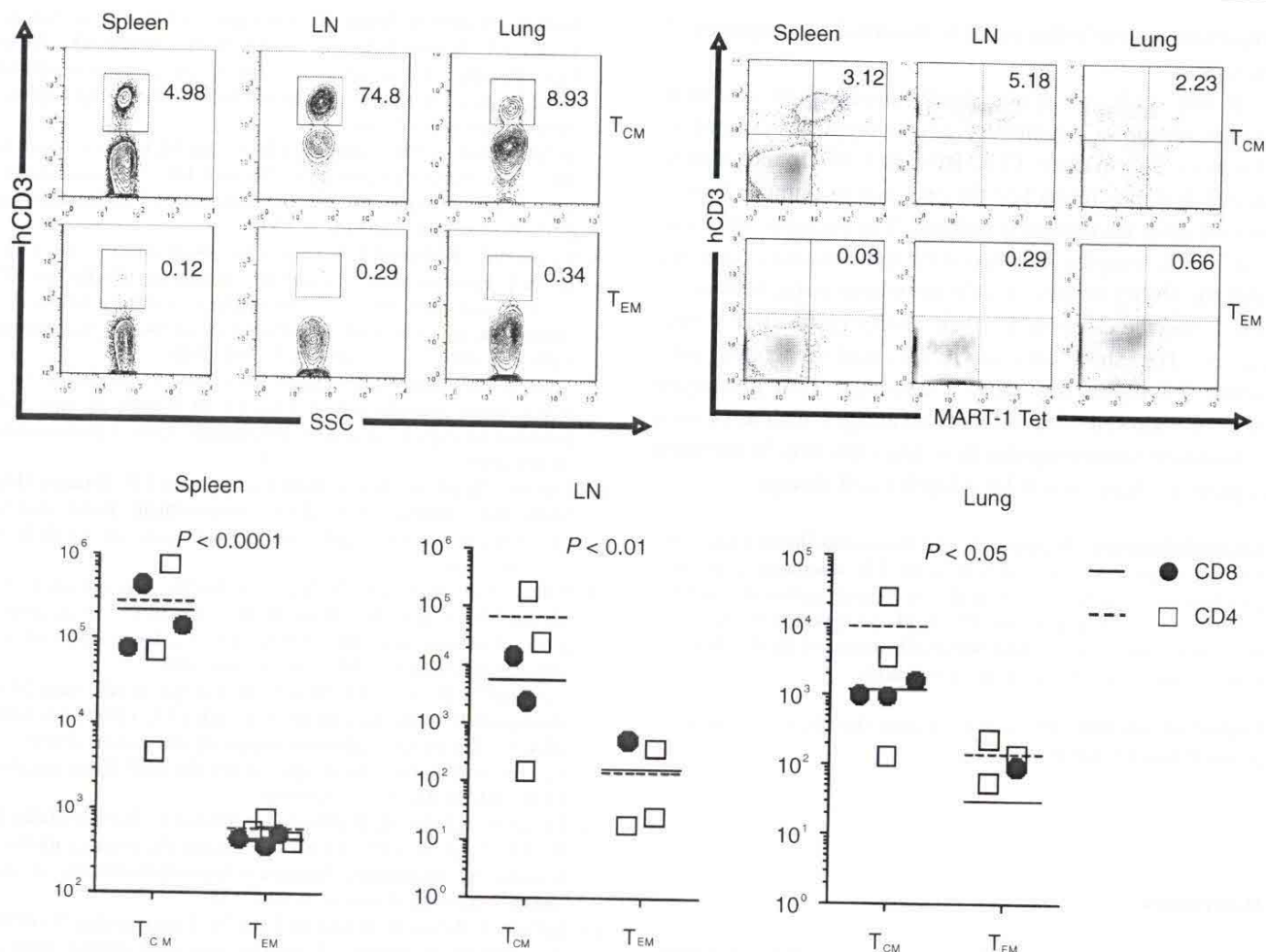


**Fig. 6** Tcm subpopulation shows an increased engraftment and persistence in NOD/SCID/ $\gamma c^{-/-}$  mice. Sorted Tem and Tcm subpopulations were injected intravenously into NOD/SCID/ $\gamma c^{-/-}$  mice without administration of IL-2. Thirty-seven days later, mice were killed and T lymphocytes were isolated from spleen, lymph nodes (LN), and lung. Upper panel, purified T lymphocytes were stained with anti-human CD3 or CD3 plus MART-1 Tetramer-PE. Data from

one representative mouse are shown in the top panels. Lower panels, for all mice the absolute number of persisting human T lymphocytes was calculated by multiplying the total lymphocyte counts from each organ by the percentage of CD3 + CD8 + or CD3 + CD4 + cells. The p value reflecting the difference in Tcm and Tem in each organ is shown

amplified a part from the heat map showing up-regulation of genes in Tcm; the possible significance of these genes in defining the role of Tcm is still under investigation. Some of the genes up-regulated in Tcm cells include IRX3 [46], a member of the Iroquois homeobox gene family that plays a role in an early step of neural development; GATA3 [47], a transcriptional activator, which binds to the enhancer of the T-cell receptor alpha and delta genes; ITGB1 [48], a member of the integrin family, which is involved in cell adhesion and recognition in a variety of processes including embryogenesis, homeostasis, tissue repair, immune response, and metastatic diffusion of tumor cells; TNFRSF4 [49], a member of the TNF-receptor superfamily that promotes the expression of apoptosis inhibitors BCL2 and BCL2L1/BCL2-XL; and lastly, LILRB3 [50], an immunoglobulin-like receptor that is thought to control

inflammatory responses and cytotoxicity to help focus the immune response and limit autoreactivity (Fig. 5).

This molecular signature was confirmed at the protein level where Tcm and Tem produced more IFN $\gamma$  (and less IL-2) compared with Tn. Due to the lack of GZMB and GZMH (Fig. 5), Tn cells had less potential to kill target cells compared with Tcm and Tem. Most significantly, when the in vitro generated anti-tumor T lymphocytes were sorted into Tem and Tcm subpopulations and infused into NOD/SCID/ $\gamma c^{-/-}$  mice [51, 52], Tcm showed increased engraftment and persistence in spleen, LN, and lung compared with those of Tem (Fig. 6). Since CD62L acts as a homing receptor, it may have helped Tcm to enter secondary lymphoid tissues and achieved sustained engraftment. Currently, we are investigating the role of CD62L on surface of human T lymphocytes, as it might represent an

important molecule that could be modified for adoptive cell therapy.

In this study, we systematically analyzed in vitro generated anti-tumor T lymphocytes phenotypically defined by differentiation markers CD45RO and CD62L and demonstrated that they mimicked the outcome of immune T cells in vivo after encountering antigen. The presence of a second T-cell receptor introduced by gene transfer does not alter the ability of these T cells to behave as memory cells, which may be essential for their ability to effectively treat cancer. The short-term in vitro culture generated anti-tumor T lymphocytes show a dominant Tcm phenotype that are endowed with intrinsic advantages such as in vivo persistence, suggesting that Tcm-like cells may be the most appropriate type of cell for adoptive cell therapy.

**Acknowledgments** We thank Arnold Mixon and Shawn Farid in the FACS laboratory and all members in the TIL laboratory at the Surgery Branch for providing technical support and maintenance of PBL and tumor cells from patients. This work is supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

**Conflict of interest** The authors declare that they have no competing financial interests.

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