ORIGINAL ARTICLE

Modulating the differentiation status of ex vivo-cultured anti-tumor T cells using cytokine cocktails

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Abstract The genetic modification of CD8+ T cells using anti-tumor T-cell receptors (TCR) or chimeric antigen receptors is a promising approach for the adoptive cell therapy of patients with cancer. We previously developed a simplified method for the clinical-scale generation of central memory-like (Tcm) CD8+ T cells following transduction with lentivirus encoding anti-tumor TCR and culture in the presence of IL-2. In this study, we compared different cytokines or combinations of IL-2, IL-7, IL-12, IL-15, and IL-21 to expand genetically engineered CD8+ T cells. We demonstrated that specific cytokine combinations IL-12 plus IL-7 or IL-21 for 3 days followed by withdrawal of IL-12 yielded the phenotype of CD62L^{high}CD28^{high} CD127^{high}CD27^{high}CCR7^{high}, which is associated with less-differentiated T cells. Genes associated with stem cells (*SOX2*, *NANOG*, *OCT4*, and *LIN28A*), were also up-regulated by this cytokine cocktail. Moreover, the use of IL-12 plus IL-7 or IL-21 yielded CD8 T cells showing enhanced persistence in the NOD/SCID/γc−/− mouse model. This defined cytokine combination could also alter highly differentiated TIL from melanoma patients into cells with a less-differentiated phenotype. The methodology that we developed for generating a less-differentiated anti-tumor CD8+ T cells ex vivo may be ideal for the adoptive immunotherapy of cancer.

Keywords CD62L · TCR gene therapy · T cell receptor · Central memory cells · Effector memory cells · Tumor immunity

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Introduction

Antigen-specific CD8+ T cells can be used to treat cancer and prevent infections in humans [1–5]. Adoptive cell transfer (ACT) using tumor-infiltrating T lymphocytes (TIL) is the most effective immunotherapy for patients with advanced metastatic melanoma, reaching a 72 % objective response rate [1, 5, 6]. However, the requirement for identifying pre-existing tumor responsive cells limits its broad application. The genetic engineering of peripheral blood-derived T cells with anti-tumor TCRs using gamma-retroviruses readily renders autologous PBL from any patient into tumor-killing T lymphocytes in vitro with resulting clinical responses [7–9].

We previously reported a methodology that combined lentiviral vector transduction of CD8+ T cells with plate-bound anti-CD3 antibody and IL-2 for activation that yielded large numbers (>1 × 10^9) of TCR-engineered T cells within 12 days, and the majority of these cells displayed a central memory-like phenotype with pronounced in vitro anti-tumor activities [10]. It is well established that the phenotype of ex vivo-cultured T cells can be modulated by the duration of culture, the addition of pharmaceuticals, or the particular cytokine used for expansion [3, 11–14]. Cytokines sharing the common γ-chain receptor (γc) include IL-2, IL-4, IL-7,

IL-9, IL-15, and IL-21. These proteins play a variety of important roles during the immune response in vivo including long-term T-cell memory formation [15], and it has been reported that IL-15, IL-7, and IL-21 preserve/promote a less-differentiated phenotype compared to IL-2 [16–19]. Furthermore, during in vitro dendritic cell–mediated priming or expansion of anti-tumor CD8 T cells, the presence of IL-21 not only enriches for tumor-reactive CD8 T cells, but also leads to a less-differentiated phenotype [17, 20–22].

IL-12 is a non-γc cytokine, which also plays a pivotal role in the regulation of T-cell differentiation and memory formation [23–25]. IL-12 is naturally produced by dendritic cells, macrophages, and human B-lymphoblastoid cells and is synthesized in response to antigenic stimulation. It is a T-cell-stimulating factor, which can enhance the production of IFN-γ and TNF-α from T and NK cells, and reduces IL-4-mediated suppression of IFN-γ. It has been reported that priming of naïve CD8 T cells with IL-12 selectively enhances the survival of CD8/CD62L^{high} cells and results in superior anti-tumor activity in a tolerogenic murine model [26]. Moreover, we recently reported that the synthesis of IL-12 by TCR gene–engineered T cells in the tumor microenvironment greatly enhanced eradication of established B16 melanoma tumors in the pmel adoptive cell therapy model [27, 28].

In this study, we systematically analyzed the effect of cytokines or their combinations on the differentiation and proliferation of TCR-transduced, in vitro-cultured, anti-tumor CD8 T cells. We propose a recipe for the sequential and combined use of cytokines to generate less-differentiated anti-tumor T cells. A cocktail of defined cytokine combinations not only yielded less-differentiated anti-tumor CD8 T cells, also re-programmed highly differentiated TIL into an apparently less-differentiated state.

Materials and methods

Cell culture

The PBL used in this study were obtained from healthy donors and metastatic melanoma patients seeking treatments at the Surgery Branch, National Cancer Institute. All primary human cells were obtained under clinical protocols reviewed and approved by Institutional Review Board. Briefly, PBL were collected by leukapheresis, and lymphocytes were separated by Ficoll/Hypaque cushion centrifugation, washed in HBSS, and resuspended at a concentration of 1×10^6/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). Cytokines for human IL-2, IL-7, IL-15, and IL-21 were purchased from PEPROTECH

(PEPROTECH, Rocky Hill, NJ). IL-2 was used at a concentration of 300 IU/ml and all other cytokines at 10 ng/ml. Tumor-infiltrated lymphocytes (TILs) were extracted from resected melanoma metastases from patients with advanced melanoma at Surgery Branch NCI and grown using a rapid expansion protocol (REP) as described[7, 29]. Melanoma cell lines included MART-1-positive HLA-A2$^+$ 526 or HLA-A*0201 + 624 and one MART-1-positive HLA-A*0201$^-$ 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_2 humidified incubator.

Vector 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 [30]. A lentiviral vector expressing the gp100 TCR alpha and beta chains of DMF5 TCR targeting melanoma antigen MART-1 [31] was previously described [10, 32, 33].

The day before transfection, 20×10^6 293 T cells were plated onto 150-mm^2 poly-D-Lysine-coated plates (BD Biosciences, San Jose, CA) using 15 ml of culture medium for lentivirus preparation. On the day of transfection, the medium was replaced with 15 ml fresh medium 3 h before transfection. Plasmid DNA 55 μg (transfer vector 22.5 μg, VSV-G 7.5 μg, pMDLg/pRRE 15 μg, and pRSV-Rev 10 μg) and 165 μl lipofectamine 2000 (Invitrogen) were used for transfection. 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 medium was removed, 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 $1,000 \times g$ for 10 min. Supernatant containing viruses was titered using a p24 kit (ZeptoMetrix, Buffalo, NY) and was either used directly or stored at -80 °C.

Plate-bound OKT3 activation and genetic engineering of positively selected CD8+ T cells

The detailed methodology was described previously [10]. Briefly, four milliliters of PBS containing 1 μg/ml OKT3 was added to non-tissue culture 6-well plates overnight at 4 °C and blocked with PBS containing 2 % FBS for 30 min at room temperature. At day 0, positively selected CD8+ T cells were washed twice with PBS, and 5×10^6 cells in AIM-V medium containing 5 % FBS (300 IU/ml

IL-2) were added to wells of plate-bound OKT3 plates followed by centrifugation at $1,000 \times g$ for 10 min. On day 1, cells were collected and washed twice with PBS and resuspended in 1 ml of vector supernatant (MOI, 2–5) per 1×10^6 cells, which were added to one well of a 24-well plate and centrifuged $1,000 \times g$ for 2 h. Six hours after transduction, feeder cells from three donors PBMC were 40 Gy irradiated and added to wells of 24-well plates containing transduced cells at a ratio of feeder to CD8+ (10:1) in the presence of OKT3 (30 ng/ml) and IL-2 (300 IU/ml). On day 2, the medium was replaced with AIM-V containing 5 % human serum and 300 IU/ml IL-2 for extended culture. On day 4, cells were split and cytokines combinations were added accordingly.

FACS analysis

In vitro-cultured cells were washed twice with PBS, and the viability of cells was determined by a combination of forward-angle light scatter and PI staining. Cell surface expression of CD3, CD4, CD8, CD27, CD127, CCR7, CD28, CD70, CD137, CD57, CD62L, and CD45RO was measured using fluorescein isothiocyanate (FITC), APC, PE-CY-7, APC-CY-7, phycoerythrin (PE)-conjugated antibodies (BD Biosciences, San Jose, CA). MART-1: 27–35 tetramer 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 Biosciences). Six-color analysis was carried out on a Canto I or Canto II instrument (BD Biosciences) with automatic compensation. All FACS data were analyzed using FlowJo 8.1.1 software (Tree Star, Ashland, OR).

Measurement of lymphocyte reactivity to antigen

Transduced CD8 effector cells (1×10^5) were co-cultured with melanoma lines (1×10^5) 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 IFNγ 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 the mean ± SD of triplicate cultures or from multiple donors.

Real-time PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed using the Thermo-Script RT-PCR system (Invitrogen). All PCR reactions were performed using an ABI 7500 FAST real-time PCR system instrument (Invitrogen). All TaqMan probes and

reagents were purchased directly from Applied Biosystems (Invitrogen) and TaqMan β-actin control reagents kit was used for normalization.

Engraftment of Tem and Tcm in NOD/SCID/γc −/− mouse model

The in vitro-generated anti-tumor CD8 T cells were cultured using a cocktail of defined combinations of cytokines as described in the text. At day 10, 1×10^6 CD8 T cells mixed with 3×10^5 unmanipulated CD4 T cells isolated from the same donor were suspended in PBS and injected intravenously into NOD/SCID/γc−/− mice as previously described [34, 35]. Forty days later, mice were killed, and lymphocytes were extracted from lymph nodes (LN) and washed twice before FACS analysis.
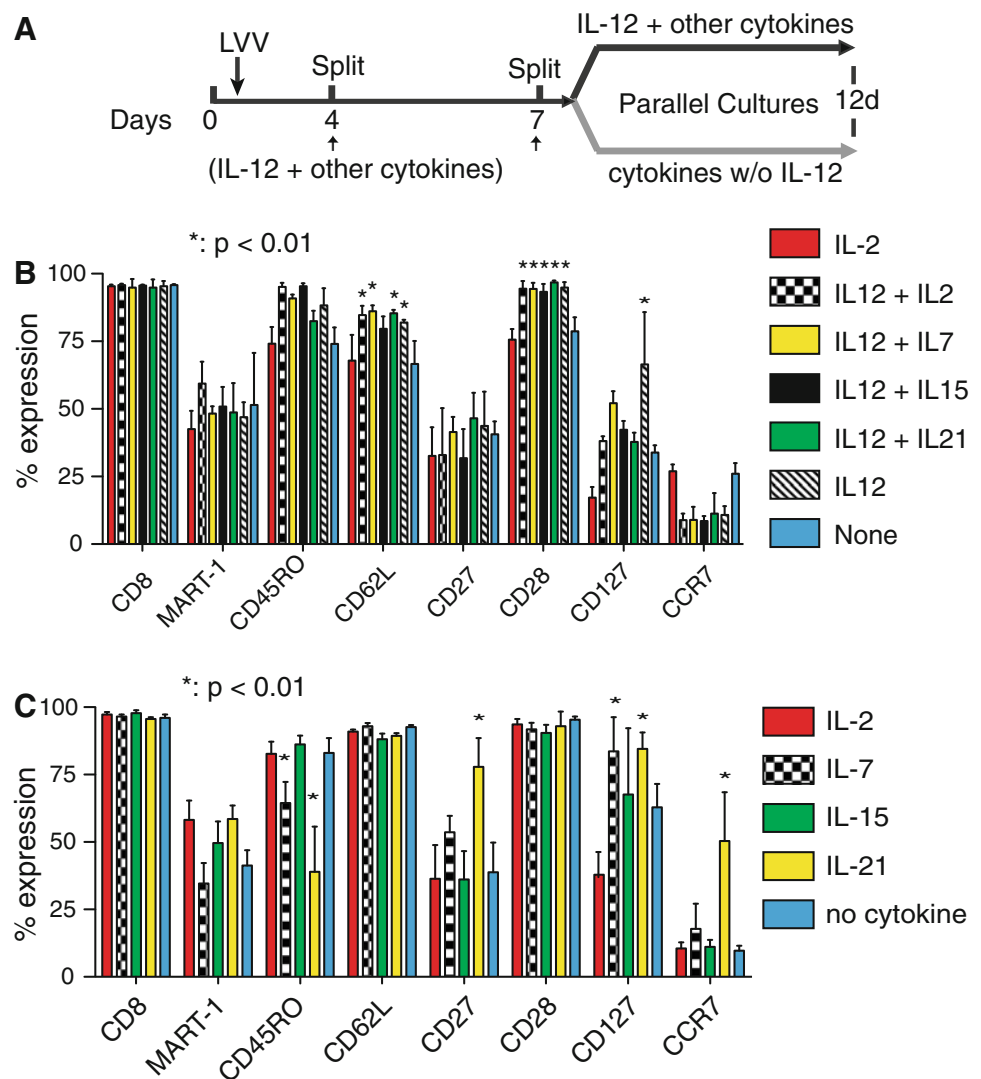
Results

A cocktail of defined cytokine combinations affects the phenotype and function of in vitro-cultured anti-tumor CD8+ T cells

Multiple pilot clinical studies have suggested that the engineering of T cells with anti-tumor antigen-specific receptors (TCR or CAR) can mediate regression of established tumors in cancer patients [7–9, 36, 37]. The target cell for the experiments reported herein was CD8+ T cells. For stimulation, we used plate-bound OKT3 as we had previously demonstrated that this was superior to CD3/CD28 bead stimulation for expansion of lentiviral vector–engineered CD8+ T cells [38]. As illustrated in the schema (Fig. 1a), all cultures were initiated in the presence of IL-2, but starting at day 4, the cells were supplemented with different cytokines or their combinations, and then at day 7, the cultures were split into two parallel cultures in which one arm kept the same combination of cytokines plus IL-12, while in the other arm IL-12 was removed (Fig. 1a). After a total of 12 days of in vitro culture, the phenotype of these two parallel cultures was analyzed (Fig. 1b, c). Representative FACS plots from three donors were as illustrated in Supplementary Fig. 1. Compared to cells cultured exclusively in IL-2, all cytokine combinations (except for the combination of IL-12 + IL-15 and control cultures) led to a significant ($p < 0.01$) increase in CD28+ cells, and the combinations of cytokines with IL-12 also led to a higher level of CD62L expression (Fig. 1b, $p < 0.01$).

Removal of IL-12 at day 7 resulted in a significant down-regulation of CD45RO, and a significant up-regulation of CD127 in the IL-7 and IL-21 groups (Fig. 1c). Markers CD27 and CCR7 were higher in the group

switched to IL-21 alone (Fig. 1c). In general, the in vitro-generated anti-tumor CD8+ T cells cultured in IL-12 plus other cytokines showed similar function when co-cultured with antigen-matched tumor lines (Supplementary Fig. 2). In cultures where IL-12 was withdrawn at day 7, the IL-21-treated group showed a reduced production of IFN-γ and IL-2 in co-culture assays (Supplementary Fig. 2). Except for the IL-21-treated group, all cultures underwent a similar fold expansion during 12 days of in vitro culture (Supplementary Fig. 2).

The resultant phenotype of CD8+ T cells grown in cultures where IL-12 was withdrawn at day 7 was investigated further by repeating the experiment in three additional donors (without the IL-15 group). FACS analysis demonstrated that compared to cells grown in IL-2 alone, the combination of IL-12 plus IL7 or IL21 generated CD62L^high^CD28^high^ CD127^high^CD27^high^CCR7^high^ anti-tumor CD8 T cells (Fig. 2b); moreover, the increase in

markers CD28, CD127, and CD62L was also seen at the mRNA level (Fig. 2c). The differentiation status of these cells was further analyzed by determining the mRNA levels for stem cell-associated genes and genes associated with T-cell ontogeny. In the group cultured with IL-21, we observed a significant increase in stem cell–associated genes (SOX2, NANOG, OCT4, and LIN28A) and a decrease in FOXP3 (Fig. 2c). The only effector T-cell gene seen to increase was IFN-γ, which was significantly up-regulated at the level of mRNA in cultures treated with IL-2 plus IL-12 (Fig. 2c). Co-culture of these cells with matched melanoma lines demonstrated increased production of IFN-γ (Fig. 3a) and IL-2 (Fig. 3b) in the IL-12 plus IL-7 and IL-2 plus IL-12 groups and reduced cytokine production (at day 13) in the IL12 plus IL-21 group. Cell growth was again similar with the exception of the IL-12 plus IL-21 group, which did not continue to expand (Fig. 3c).

Fig. 2 Cytokine combinations lead to a less-differentiated phenotype in vitro. **a** Schema of cytokine in vitro culture. All cultures were initially stimulated and maintained in IL-2 until day 4. LVV, lentiviral vector transduction; w/o, without. **b** Phenotype of in vitro-cultured anti-tumor CD8 T cells grown in defined cytokine combinations at day 12. Following FACS, the percentage of each marker was calculated (*left*) and the MFI presented (*right*). **c** Gene expression of in vitro-cultured anti-tumor CD8 T cells. Total RNA from the cells was extracted and reverse-transcribed; the level of mRNA representing each candidate gene was measured using Q-RT-PCR. The level of mRNA at IL-2 group was set up as 1; the level of mRNA from other groups was normalized accordingly. The mean ± SD from three donors was calculated, and *t* test was used for statistical analysis. An *asterisk* indicates $p < 0.05$ compared to the IL-2 group
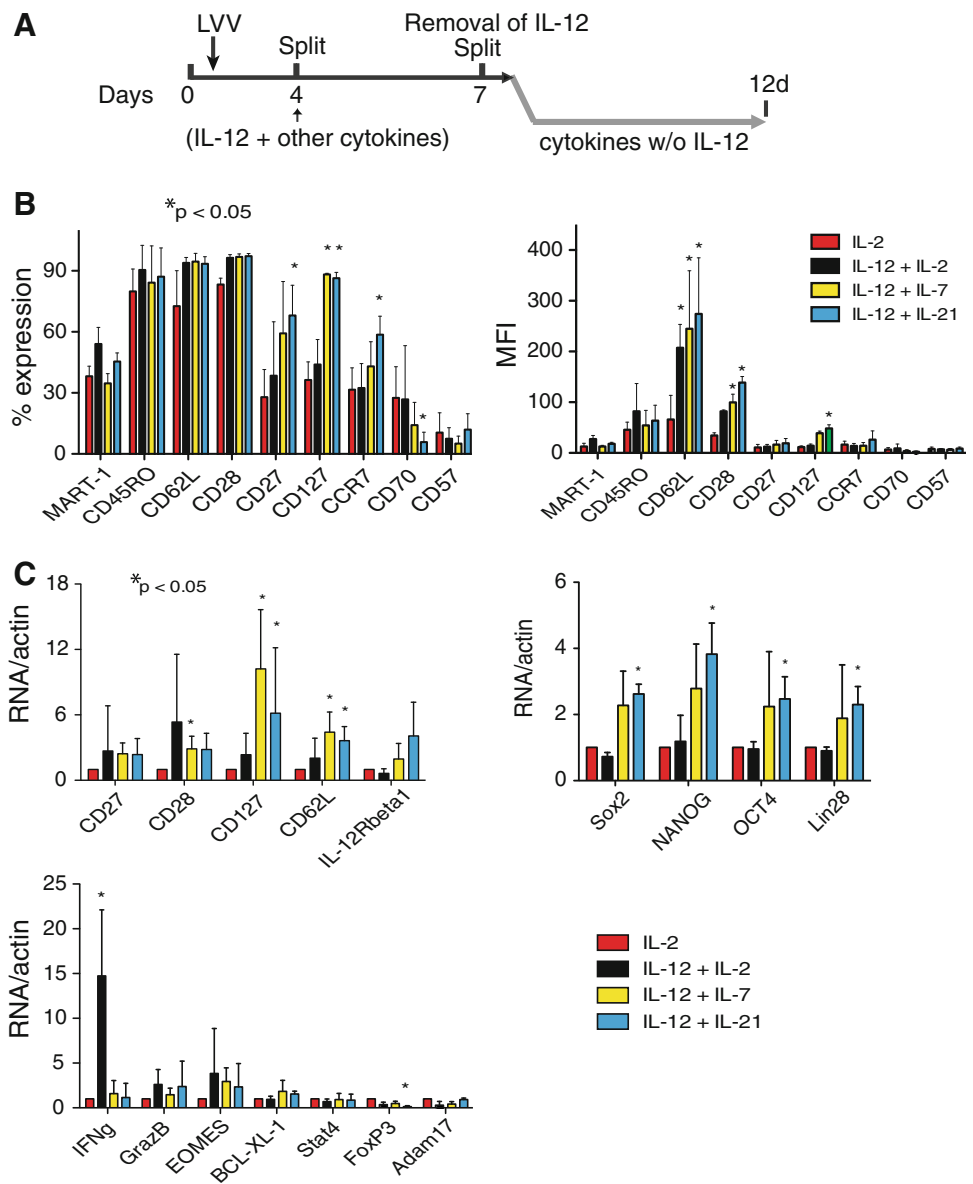
**CD8 T cells primed with defined cytokine combinations show enhanced engraftment in NOD/SCID/γc$-/-$ mice**

We have previously reported on the use of the NOD/SCID/γc$-/-$ mouse model to monitor the engraftment of in vitro-generated anti-tumor T cells from melanoma patients [35]. We generated anti-tumor CD8 T cells using the defined cytokine regimen of IL-12 plus IL-7 or IL-21 between days 4 and 7, followed by removal of IL-12 and culture until day 10. The cells were harvested, washed, and injected intravenously into the mice. Forty days later, mice were killed and lymphocytes extracted from lymph nodes (Fig. 4). We detected the enhanced engraftment of human CD8 T cells in LN from groups treated with IL-12 plus IL-7 or IL-21

compared to the IL-2-treated group, and the difference of persistent cells was statistically different when calculating the number of viable human CD8 T cells (Fig. 4).

Cytokine combinations induce a less-differentiated phenotype in TIL from melanoma patients

TIL used for ACT are generally highly differentiated late-stage effector cells with a phenotype of CD27low, CD28low, CD45RA$-$, CD62L$-$, CCR7$-$, and CD127low [39]. Because of the substantially phenotypic difference resulting from the use of cytokines during in vitro culture of TCR gene-modified PBL, we tested whether the defined cocktail of cytokine combinations could also influence the phenotype

Fig. 3 Function of cytokine-treated anti-tumor antigen CD8 T cells. T cells grown as described in the schema in Fig. 2a and then tested for anti-tumor reactivity at day 10 or 13 in the different cytokine combinations as shown. Co-cultures were with melanoma lines (mel 526, MART-1 +/HLA-A2+ and mel 938, MART-1 +/HLA-A2−) followed by determination of **a** IFN-γ and **b** IL-2. **c** The number of viable cell in each cytokine combination was determined and the fold expansion plotted
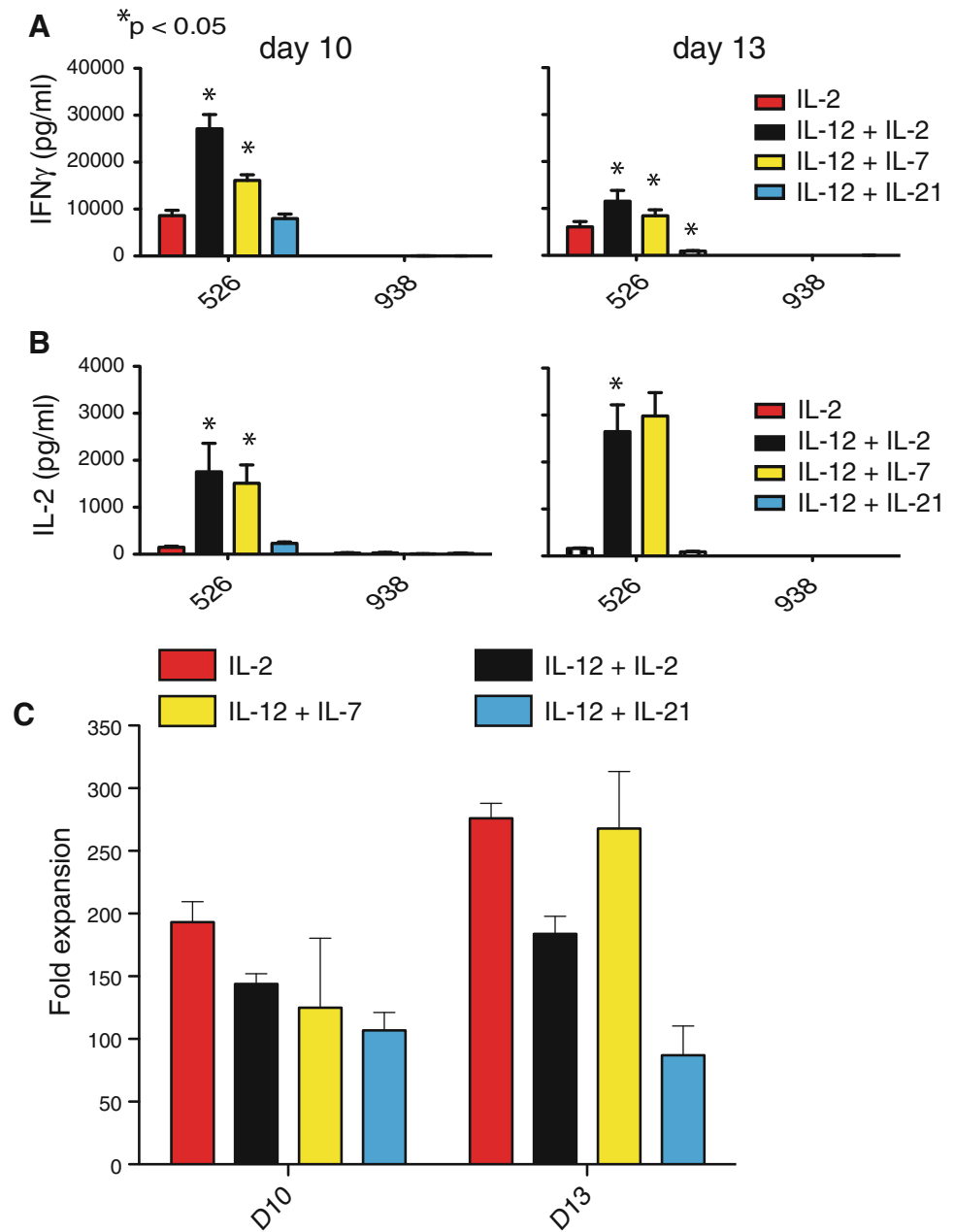
of TIL. At day 15 following rapid expansion, we took TIL and cultured them in media supplemented with the cytokine combinations as shown for 6 days (Fig. 5a). At the end of 6 days in culture, cells underwent one- to threefold expansion, and the percentage of viable cells from 6 donors ranged from 60 to 85 % (Fig. 5b). The IL-21-treated cells showed the lowest viable cell number and the least expansion (Fig. 5b). TILs cultured under these different conditions showed dramatically different appearances. In cultures of TIL grown in IL-7 and IL-21, the cells exhibited a rounded morphology and grew in a diffuse pattern compared to cells in the IL-2 groups, which grew in clusters (Fig. 5c).

Unexpectedly, the 6-day cytokine treatment in vitro led to a substantially phenotypic change in TIL. The expression of CD62L and CD127 increased significantly in three groups supplemented with IL-12, while the markers reflecting late-stage effector cells CD70 [35] and CD57 [40] significantly decreased in IL-7- and IL-21-treated groups (Fig. 6a). In some circumstances, the phenotypic changes were consistent with changes at mRNA level, that is, CD127 and CD62L, etc. (Fig. 6b). We also observed a significant enhancement of stem cell–associated genes (*SOX2*, *NANOG*, and *OCT4*) in groups supplemented with IL-7 or IL-21 (Fig. 6b).
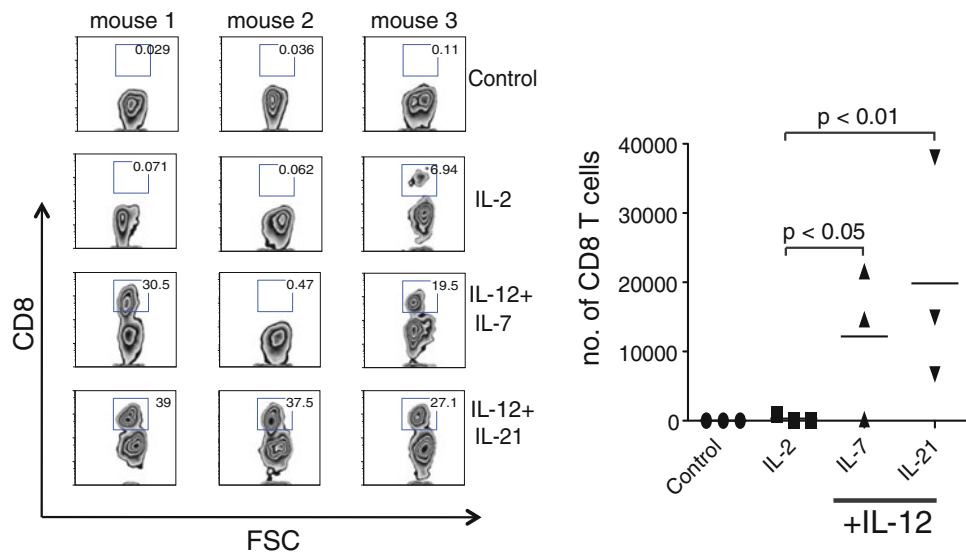
Fig. 4 CD8 T cells cultured with IL-7 and IL-21 show enhanced engraftment in NOD/SCID/γc−/− mice. The CD8 T cells were transduced and maintained in the presence of IL-2. At day 4, the cells were split, cultured in IL-2 alone or IL-12 plus IL-7 or IL-21 until day 7, at which time the cells were split and the cultures continued without IL-12. At day 10, 1×10^6 CD8 T cells mixed with 3×10^5 unmanipulated CD4 T cells isolated from the same donor were injected intravenously per mouse. Forty days later, mice were killed, and lymphocytes were extracted from lymph nodes. FACS was performed using anti-human CD8 antibody on *left*; the total number of engrafted human CD8 were calculated and plotted on *right*. Control group indicates only CD4 T cells were injected. The mean ± SD from three mice was calculated

Fig. 5 Effect of cytokine cocktails on TIL. **a** The schematic illustration of cytokine addition during 6-day in vitro culture. TILs were rapidly expanded until day 15 of growth, then harvested and washed, and the cytokines or their combinations were added as depicted. The concentration of IL-2 was 300 IU/ml, and the other cytokines were at 10 ng/ml. At day 3, the supernatant from the TIL culture was removed, and other cytokines were added without the use of IL-12. **b** The viability and fold expansion during 6-day in vitro culture. **c** The morphological variation of TILs cultured with different combinations of cytokines at day 6. Shown are representative photomicrograph fields, *scale bar* indicates 50 μm
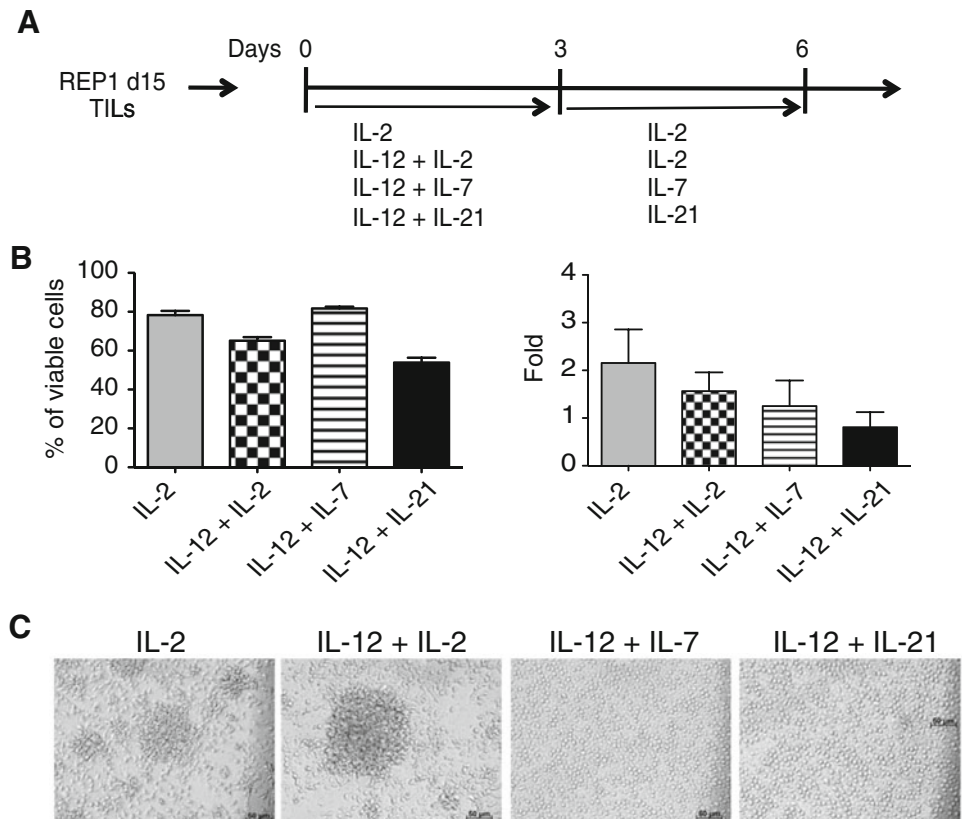
Fig. 6 The phenotype of TIL grown with different combinations of cytokines. **a** The phenotype of TILs at day 6 was analyzed by FACS using a panel of antibodies as shown. **b** Gene expression of cytokine-cultured TILs at day 6. The total RNA from the cells was extracted and reverse-transcribed; the level of mRNA representing each candidate gene was measured using Q-RT-PCR assay. The level of mRNA at IL-2 group was set up as 1; the level of mRNA from other groups was normalized accordingly. The mean ± SD from six donors was calculated. An *asterisks* indicate $p < 0.01$ compared to the IL-2 group
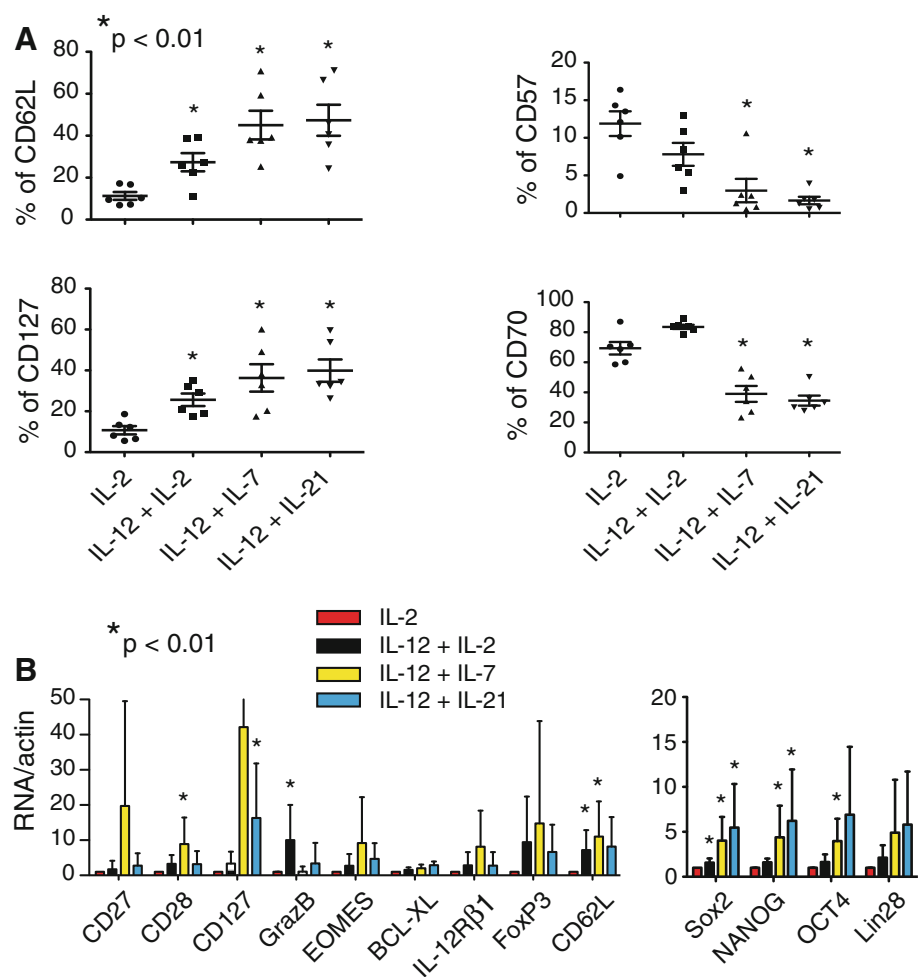
Discussion

The ultimate goal of adoptive cell transfer is to eliminate tumor cells and the generation of anti-tumor immunological memory. We have reported that in vitro genetically engineered anti-tumor T cells mimic in vivo-antigen-experienced T cells exhibiting distinct subsets, including Tcm-like cells [35]. Cytokines have been well studied for their ability to affect the in vitro and in vivo properties of T cells, but it is unlikely that a single cytokine can bestow onto T cells the multitude of properties associated with effective adoptive cell therapy. We thus sought to investigate both different cytokine combinations and their duration of addition on the in vitro properties of TCR gene–engineered human T cells. Cytokines sharing the γc receptor are required for the development or maintenance of memory T cells and have very diverse functions, but only IL-2 has undergone extensive study in humans. Antigen-specific CD8+ T cells from mice can be induced to differentiate during in vitro culture in the presence of IL-2. Depending on the length of culture, cells can have a phenotype associated with naïve, early effector,

intermediate effector, and late effector cells [3]. Ironically, despite having the strongest anti-tumor effects in vitro, the highly differentiated effector T cells were significantly less effective in vivo compared to T cells at their early effector stage. In practice, the criteria used to select T cells for clinical application included IFNγ release or in vitro cytotoxicity, which can be negatively correlated with in vivo anti-tumor efficacy in mice.

While the role of cytokines is diverse, it has been reported that IL-7 and IL-21 are superior to IL-2 and IL-15 in promoting human T-cell-mediated rejection of systemic lymphoma in immunodeficient mice [19]. Furthermore, we demonstrated that IL-21 prevented differentiation of CD8+ T cells in vitro and the treatment with IL-21-cultured CD8+ T cells robustly eradicated large established tumors in a mouse model [13]. More recently, we reported significantly enhanced anti-tumor reactivity of IL-12 gene-modified T cells [27, 28]. Based on our data reported herein, we proposed a recipe for a defined cytokine combination to be used in the culture of retroviral vector gene–engineered T cells. Specifically, following the initial stimulation in culture with IL-2 as described [38], medium is changed to IL-12 plus

IL-7 or IL-21 for 3 days followed by culture without IL-12 for another 3–5 days. This cytokine cocktail reproducibly yielded TCR gene–engineered CD8 T cells displaying CD62L^{high}CD28^{high} CD127^{high}CD27^{high}CCR7^{high} phenotype associated with less-differentiated cells. Moreover, these cells with a less-differentiated phenotype engrafted significantly better than the cells grown exclusively in IL-2 following ACT in NOD/SCID/γc−/− mice.

We chose to adopt the culture conditions where IL-12 was removed at day 7 as these conditions in combination with IL-7 or IL-21 yielded more CD8 + T cells with markers CD27 and CD127 (Fig. 1c). In TIL used for ACT of patients with metastatic melanoma, the factors associated with objective response included longer telomeres of the infused cells, increased numbers of CD8+/CD27+ cells, and the persistence of the infused cells in the circulation at 1 month [5]. In addition, we previously reported that following administration of tumor antigen-specific T cells, a rapid up-regulation and continued expression of CD127 was an important factor in intermediate and long-term T-cell survival [39]. Moreover, two months after transfer of T cells with a late effector cell phenotype (CD27lowCD28lowCD62L-CCR7-), there was a transition to the development of long-term, melanoma-reactive memory CD8+ T cells in vivo showing a phenotype of CD27+CD28+ cells [39]. Recently several groups have reported that IL-21 can have profound effects on tumor antigen-specific T cells, including increases in CD62L, CD28, CD27, and CD127 [13, 20, 21]. Our observations that genetically engineered CD8+ T cells can be similarly programmed by culture conditions including cytokines IL-7 or IL-21 could have immediate impact on ACT trials using TCR- or CAR-engineered T cells.

One of the unexpected observations in this report was the finding that highly differentiated TIL can undergo phenotypic change by cytokines (Figs. 5, 6). When we cultured TIL with a terminally differentiated cell-like phenotype using the recipe of combined cytokines for 6 days, we observed a significant up-regulation of CD62L, and CD127 and a decrease in CD70 and CD57. This observation along with the induction of transcripts associated with stem cells and a distinct morphological change suggests that TIL can be re-programmed in vitro. Using the strategy pioneered by Yamanaka, multiple investigators have reported that mature T cells can be re-programmed to induced pluripotent stem cells (iPS) while retaining their rearranged T-cell receptor [41–43]. It is thus conceivable that these iPS could then be differentiated to functional naïve T cells (Tn) or T stem cell memory cells (Tscm) that possess enhanced anti-tumor reactivity[34, 44].

Our study suggests that cytokine combinations can be used to mediate the differentiation of in vitro-cultured human anti-tumor CD8+ T cells. In addition, the recipe of cytokines we proposed for generating less-differentiated CD8 + T cells in vitro was also capable of re-programming the late effector cells from TIL (although these experiments do not rule out the possibility that culture selectively enriched for less-differentiated TIL). As more and more cytokines become commercially available and are provided as GMP reagents, the optimized utilization of cytokines, such as we propose here, may lead to the design of versatile protocols for better ACT of cancer.

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

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