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Clinical-scale selection and viral transduction of human naïve and central memory CD8⁺ T cells for adoptive cell therapy of cancer patients

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Abstract

The adoptive transfer of lymphocytes genetically engineered to express tumor-specific antigen receptors is a potent strategy to treat cancer patients. T lymphocyte subsets, such as naïve or central memory T cells, selected in vitro prior to genetic engineering have been extensively investigated in preclinical mouse models, where they demonstrated improved therapeutic efficacy. However, so far, this is challenging to realize in the clinical setting, since good manufacturing practices (GMP) procedures for complex cell sorting and genetic manipulation are limited. To be able to directly compare the immunological attributes and therapeutic efficacy of naïve (T_N) and central memory (T_{CM}) CD8⁺ T cells, we investigated clinical-scale procedures for their parallel

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Conflict of interest Mario Assenmacher is employee of Miltenyi Biotec GmbH and Alexander Scheffold works as a consultant for Miltenyi Biotec GmbH. All other authors do not have any conflicts to disclose.

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selection and in vitro manipulation. We also evaluated currently available GMP-grade reagents for stimulation of T cell subsets, including a new type of anti-CD3/anti-CD28 nanomatrix. An optimized protocol was established for the isolation of both CD8⁺ T_N cells (CD4⁻CD62L⁺CD45RA⁺) and CD8⁺ T_{CM} (CD4⁻CD62L⁺CD45RA⁻) from a single patient. The highly enriched T cell subsets can be efficiently transduced and expanded to large cell numbers, sufficient for clinical applications and equivalent to or better than current cell and gene therapy approaches with unselected lymphocyte populations. The GMP protocols for selection of T_N and T_{CM} we reported here will be the basis for clinical trials analyzing safety, in vivo persistence and clinical efficacy in cancer patients and will help to generate a more reliable and efficacious cellular product.

Keywords

Immunotherapy; Adoptive cell therapy; T cell subsets; Naïve and central memory enrichment

Introduction

Through the adoptive transfer of genetically engineered lymphocytes, it is possible to mediate objective cancer regression in human patients with metastatic melanoma and other advanced cancers [1–3]. Adoptive T cell therapy relies on the in vitro expansion of endogenous or engineered cancer-reactive T cells, which are harvested from cancer patients, manipulated, and then reintroduced to provide protective antitumor immunity. Tumor regression using genetically retargeted peripheral blood lymphocytes has now been reported for patients with melanoma, sarcoma, colon cancer, lymphoma and leukemia [4–8]. Numerous clinical trials are currently accruing patients to extend the range of antigens and tumor histologies that can be targeted [9–11]. However, the efficacy of adoptive immunotherapy in humans is often limited and the critical parameters for the in vivo efficacy remain poorly defined. There can also be variable toxicities associated with this approach [7, 12, 13]. Some efforts to improve this treatment focus on the lymphocyte population that is used for genetic retargeting.

Peripheral blood T cells represent a complex mixture of different functional attributes and differentiation states [14]. Selecting a more uniform lymphocyte population for retargeting could standardize and improve treatment outcomes. One way to improve efficacy could be to improve in vivo survival time of transferred T cells by selecting T cells with higher self-renewal capacity. Studies with human tumor infiltrating lymphocytes (TIL) demonstrate that terminally differentiated effector memory cells fail to persist and are poor mediators of tumor regression [1, 15]. Some reports in the last few years have shown in different animal models how it is possible to improve antitumor response [16] and persistence of transferred cells by using engineered T cell subsets [17]. Alternately, different PBL subsets contain TCR repertoires with different complexities. It has been shown in humans that the intrinsic response of T_{CM} to viral antigen boosts antitumor response of the introduced tumor-specific receptor-mediating T cell killing [10]. At the same time, the reduced complexity of the T_{CM} repertoire could reduce the risk of inappropriate TCR pairing and off-target reactivity [18]. Although it is not clear at present which subset represents the optimal target for adoptive immunotherapy in human beings, these data support investigation of a less differentiated and

more uniform T cell subset such as stem-cell-like memory T cells, T_N or T_{CM}, instead of the whole PBL population for T cell engineering.

The main problem for translation of this concept from bench to clinic and in particular for the transfer of successful therapeutic strategies among different clinical centers is the lack of an appropriate sorting protocol for clinical-grade isolation of CD8⁺ T_N and T_{CM} cells. Similarly, reliable procedures and reagents for the activation and transduction of the isolated subsets under GMP conditions are missing. Current standard protocols for in vitro activation and retroviral transduction of T cells are typically based on stimulation with soluble GMP anti-CD3 (αCD3) antibody together with accessory cells, such as autologous antigen presenting cells (APC), providing cross-linking activity for the soluble antibodies and further co-stimulatory signals. This system is strongly dependent on the quality of the accessory cells and, in addition, requires high cell culture volumes and corresponding high amounts of viral supernatant, which is currently a major cost item for the cell production process. Isolated T cell subsets can directly be activated and transduced under defined in vitro conditions using GMP αCD3 and αCD28 antibodies immobilized on large solid surfaces like the cell culture dish or cell-sized particles [19, 20]. These systems are time-consuming, difficult to standardize and control under GMP conditions, and large particles require removal prior to injection of cells into patients. In the present study, we have developed a sorting strategy for the enrichment of T_N and T_{CM} CD8⁺ T cell subsets using GMP-compliant procedures and reagents. For the activation of CD8⁺ T cell subsets, we introduce a new flexible nanomatrix (TransAct reagent) conjugated with αCD3 and αCD28 antibodies with superior properties and easier handling in particular in a closed systems under GMP conditions. We show that T_N and T_{CM} cells sorted from blood of metastatic melanoma patients can efficiently be expanded and transduced under these conditions. Importantly, T_N and T_{CM} cells show minimal effector differentiation and lower levels of markers correlating with terminal differentiation when stimulated with TransAct or αCD3/αCD28-conjugated micron-sized particles (MACSiBeads). Taken together, our study provides a well-defined technological platform for the GMP sorting, expansion and genetic engineering of defined T cell subsets for use in clinical trials to treat patients with cancer.

Materials and methods

CliniMACS selection

Fresh leukapheresis samples from patients with metastatic melanoma on National Cancer Institute Institutional Review Board-approved clinical trials were used for all studies. PBMC were enriched over a Ficoll-hypaque step gradient to remove residual red blood cells and neutrophils and washed twice in PBS. For T_N and T_{CM}, $2-4 \times 10^9$ PBMC were utilized in each lymphocyte subset separation.

T_N cells were enriched starting with PBMC incubated for 30 min on a rotator at room temperature (RT) with αCD4 CliniMACS Microbeads (Miltenyi Biotec). Cells were washed by centrifugation, and labeled cells were resuspended in 100 ml of CliniMACS buffer with 20 % human albumin (Plasbumin-25 25 %, USP Talecris Biotherapeutics) (buffer) and transferred to a collection bag. The bag was connected to a CliniMACS Selection System, and CD4⁺ cells were depleted using the Depletion 2.1 program with the large-scale tubing

set. The target fraction was then labeled with α CD62L Microbeads for 15 min in cold buffer, washed by centrifugation and connected to the CliniMACS Selection System for enrichment with the Enrichment 3.2 program using the standard tubing set. The target fraction was then labeled with α CD45RA.biotin and α Biotin.MACSiBeads. The target population was enriched using a MACSiMag isolation system.

T_{CM} cells were enriched starting with PBMC incubated for 30 min on a rotator at RT with CD4 and CD45RA CliniMACS Microbeads in cold Buffer. Excess Microbeads were removed by cell washing, and labeled cells were resuspended in 100 ml Buffer. The collection bag containing the labeled cells then was connected to a CliniMACS Selection System set for the depletion 2.1 program with the large-scale tubing set. The target fraction was subsequently labeled with α CD62L Microbeads for 15 min in cold buffer, washed and connected to CliniMACS Selection System for enrichment according to program Enrichment 3.2 with a standard tubing set.

Flexible nanomatrix for T cell stimulation (TransAct)

The TransAct reagent was provided by Miltenyi Biotec. TransAct is a colloidal reagent consisting of nanoscale iron oxide crystals embedded into a biocompatible polysaccharide matrix with an overall diameter of about 100 nm. Antibodies against CD3 (clone OKT3) and CD28 (clone 15E8) are covalently attached to the matrix. The matrix can be produced under cGMP conditions, sterilized by filtration and unbound reagent can easily be removed from the cell suspension by centrifugation of the cells. In the experiments described here, the reagent was used at 0.5 μ g/ml of both antibodies conjugated in a 1:1 ratio. Anti-CD3 and anti-CD28 TransAct reagents can also be used when conjugated on separate matrices and mixed in variable ratios and concentrations to achieve optimal T cell activation.

T cell stimulation and transduction

Isolated CD8⁺ T cell subsets were stimulated either with coated α CD3 (OKT3, 10 μ g/ml) in the presence of soluble α CD28 (15E8, 1 μ g/ml) or with MACSiBeads (loaded with α CD3, α CD28 and α CD2, Miltenyi Biotec) at a 1:1 cell/bead ratio or with TransAct (0.5 μ g/ml). All cultures contained interleukin (IL)-2 (Aldesleukin, Prometheus) at 300 IU/ml throughout the duration of cell growth. The mF5 retrovirus encoding an anti-MART-1 T cell receptor (TCR) comprised of mouse alpha and beta TCR constant regions and human TCR CDR antigen combining regions [21] was used for all transductions.

Transductions were performed as previously described [22]. Briefly, lymphocytes were harvested, washed once and exposed to Retronectin and retrovirus-coated plates approximately 60 h after initial stimulation. To coat plates, clinical-grade GMP retroviral supernatant [23] was centrifuge onto 6-well Retronectin-coated (Takara Bio, Osaka, JN) plates for 2 h at 2,000g and 32 °C. Activated lymphocytes or CD8⁺ T cell-enriched subsets were then plated and centrifuged for 10 min at 1,500 rpm. T cells were transferred the following day to a second set of pre-coated Retronectin/retroviral vector tissue culture plates for second transduction. After approximately 16 h, the cells were moved from Retronectin-coated plates to tissue-culture-treated flasks (Corning). The cells were cultured for a total of 14–15 days in AIMV + 5 % human serum + 300 IU/ml IL2 and split every second day.

Flow cytometry and cytokine production assays

Cells were labeled with fluorescent antibodies against the following targets: CD8, CD4, CD45RA, CD62L, CD127, IFN γ , IL2, TNF α (all from Miltenyi Biotec); CCR7, CD27, CD45RO (BD Biosciences); and MART-1 tetramer (Beckman-Coulter, Allele HLA-A 0201, peptide MART-1, ELAGIGILTV). Intracellular cytokines were determined by coculture of transduced cells with the 624 melanoma cell line (HLA-A2⁺, MART1⁺) or 888 melanoma cell line (HLA-A2⁻, MART1⁺) in the presence of Brefeldin A (BD Bioscience). After 6 h of coculture, the cells were labeled with α CD8, fixed and permeabilized with Miltenyi Inside Stain Kit (Miltenyi Biotec). Flow cytometric data were acquired using BD FACSCanto II cytometer (BD Biosciences) and were analyzed with FlowJo Version 7.5 software (TreeStar).

Statistical analysis

Groups were compared using student *t* test with significance reported as $^*(0.01 < P < 0.05)$, $^{**}(0.005 < P < 0.01)$, and $^{***}(P < 0.005)$. Statistical results were similar with pairwise comparisons using nonparametric Mann–Whitney *U* tests (not shown). All *P* values are two tailed and are not corrected for multiple comparisons.

Error bars in graphs represent the SEM.

Results

Clinical selection of human naïve and central memory CD8⁺ T cells

Human CD8⁺ peripheral T cells can be segregated in T_N, T_{CM} and T_{EM} according to differential expression of CD45RA and CD62L [24]. CD8⁺ T naïve cells are CD45RA⁺CD62L⁺, whereas antigen-experienced T_{CM} and T_{EM} cells are CD45RA⁻ and can be further discriminated based on the different expression of CD62L (T_{CM}: CD8⁺CD45RA⁻CD62L⁺; T_{EM}: CD8⁺CD45RA⁻CD62L⁻). Based on these phenotypic characteristics, the isolation of the CD8⁺ T_N subset was achieved first by depleting CD4⁺ cells, and then by a secondary enrichment of CD62L⁺ T cells. Figure 1a shows the phenotypic analysis of T cells after each enriching step. This resulted in a CD4⁻CD62L⁺ population with purity of 86.4 ± 7.8 % among CD8⁺ cells ($n = 6$, Table 1), containing less than 0.1 % CD⁺ T cells. The cell recovery was 72 ± 27 and 75.5 ± 7.8 % for CD4 depletion and CD62L enrichment steps, respectively. From the resulting CD62L⁺ population, the T_N cells were further enriched by magnetic selection of CD45RA⁺ cells. The second positive enrichment, from the CD62L selected population, was performed using large superparamagnetic particles (MACSiBeads, diameter 3.5 μ m) to sort CD45RA⁺ cells via a standard permanent magnet without interference by the previously applied small α CD62L superparamagnetic Microbeads. The purity of the target population (TN) after CD45RA enrichment was 75.2 ± 15.5 % among CD8⁺ cells (Table 1) and the overall cell recovery for the whole procedure was 44.6 ± 16.7 %. The CD8⁻ cells remaining in the target fraction (52 ± 9 %) were mainly NK and B cells (data not shown).

CD8⁺ T_{CM} cell enrichment consisted of two Clini-MACS® Plus separation steps, first the depletion of CD45RA⁺ together with CD4⁺ cells and a subsequent enrichment of CD62L⁺

cells. This process resulted in a $CD45RA^{-}CD62L^{+}$ population (Fig. 1b) with a purity of $88 \pm 9.9\%$ among $CD8^{+}$ cells and an overall cell recovery of about $60.94 \pm 23.8\%$ for the entire procedure ($n = 7$, Table 1). The $CD8^{+}$ cells present in the target fraction ($61.8 \pm 4.8\%$) were mainly myeloid cells $CD33^{+}CD62L^{+}$ (data not shown). The Non-target fraction of T_{CM} selection was enriched in $CD8^{+}CD45RA^{-}CD62L^{-}$ cells (T_{EM}) and was used as a third subset in our in vitro experiments.

Phenotypic characterization of the selected subsets

We further analyzed the phenotype of the enriched cells (Fig. 2a). Because $CD62L$ and $CCR7$ are co-expressed in both T_N and T_{CM} and since we used $CD62L$ as key marker in the enrichment protocols, we checked the percentage of $CCR7^{+}$ cells in each subset (Fig. 2b). We found that a large proportion of T_N ($70 \pm 5.5\%$) and T_{CM} ($54 \pm 7\%$) enriched cells express $CCR7$, whereas few cells in T_{EM} subset ($13.2 \pm 2.4\%$) were positive for $CCR7$, as expected. The $CD8^{+} T_N$ fraction was mainly composed of cells expressing the $IL7R\alpha$ molecule ($CD127$) on the surface ($62 \pm 5.7\%$), whereas the $CD127^{+}$ population was significantly smaller in T_{CM} and T_{EM} samples (30 ± 3.7 and $30.7 \pm 7.3\%$, respectively; Fig. 2c). $CD27$ expression is known to positively correlate with replicative capacity and tumor response in a clinical trial of TIL Adoptive Cell Therapy (ACT) [25, 26]. $CD27$ expression was uniformly highly expressed in all subsets (Fig. 2d); however, expression was significantly higher in T_N and T_{CM} (88 ± 1.8 and $80.5 \pm 5.3\%$, respectively) compared to T_{EM} ($62 \pm 5.4\%$). Consistent with the notion that lymph node homing capacity and the acquisition of effector function are coordinately regulated [24, 27], we also analyzed expression of GranzymeA (GranzA) in the T cell subsets (Fig. 2e). As expected, only $16.7 \pm 3.6\%$ of T_N cells were positive for GranzA and this percentage increased according to the degree of cell differentiation from $42.5 \pm 6.6\%$ for T_{CM} - to $85 \pm 2.2\%$ for T_{EM} -enriched fraction. These data show that the GMP-compatible protocols described here are suitable to select highly enriched T_N and T_{CM} subsets displaying defined phenotypic characteristics, at large scale.

Optimized reagents for in vitro activation and TCR transduction of T cell subsets

Current standard protocols for in vitro activation and viral transduction of T cells typically use soluble GMP $\alpha CD3$ antibodies in the presence of autologous APC, providing cross-linking activity for the soluble antibodies and co-stimulatory signals. In our procedure for T cell subset enrichments, professional APCs were removed from the target fraction; therefore, we needed different systems to provide cross-linking of $\alpha CD3$ and costimulation for full T cell activation. For this purpose, we tested $\alpha CD3$ coated on the cell culture device plus soluble $\alpha CD28$ antibodies ($\alpha CD3 + \alpha CD28$), $\alpha CD3$ and $\alpha CD28$, linked to cell-sized particles (MACSiBeads) and new class of reagents consisting of $\alpha CD3$ and $\alpha CD28$ antibodies immobilized on flexible matrices ("TransAct"). TransAct are a GMP-compatible reagent able to fully stimulate T cells, but due to their small size behave similarly to soluble antibodies, e.g., can easily be removed by cell centrifugation. In our experiments, we compared $\alpha CD3 + \alpha CD28$ stimulation, MACSiBeads and TransAct particles with soluble $\alpha CD3$ -stimulated PBMC according to a standard protocol used historically for PBMC transduction in multiple clinical applications [4–7]. T_N -, T_{CM} - and T_{EM} -enriched $CD8^{+}$ T cell subsets were stimulated under the indicated conditions for 60 h and then transduced

with a retroviral vector encoding the well-characterized MART-1 TCR [13]. To test the relative frequency of transgene expression in these T cell subsets, we performed MHC-peptide Class I tetramer staining. All T cell subsets were efficiently transduced (>40 %) independent of the stimulatory conditions used (Online Source 1). However, it appeared that T_N have slightly lower frequency of tetramer-positive $CD8^+$ T cells when stimulated with MACSiBeads and TransAct reagent (46 ± 6.5 and 58.5 ± 9.3 %, respectively, vs 90 ± 2.3 % for $\alpha CD3 + \alpha CD28$), and also with respect to T_{CM} and T_{EM} stimulated under the same conditions. This is probably due to the delayed activation kinetics observed with these two stimulation systems (data not shown), and we are therefore currently adapting the transduction time point for the various conditions. However, 10 days after the transduction, we observed no differences with regard to expansion of the three subsets under all conditions. We observed a trend (not statistically significant) for better expansion of T cell subsets stimulated with MACSiBeads or TransAct when compared to coated $\alpha CD3 + \alpha CD28$ (Online Source 2). Some clinical applications require an additional in vitro expansion of transduced cells to achieve large cell numbers. In limited experiments with head to head comparisons of isolated T_{CM} versus bulk PBMC, purified T_{CM} expanded equivalently to bulk PBMC in a rapid expansion protocol (1,000–3,600 fold expansion, depending on the patient).

We next analyzed the phenotypic and functional characteristics of cells generated from each subset. After 13–15 days of expansion, we stained cells for CCR7, CD62L, CD57, CD27 and CD127 markers. The CD62L marker is equally retained on the surface of T_N and T_{CM} cells and better than on T_{EM} cells independent of the stimulation conditions (Fig. 3a). In contrast, CCR7 is efficiently expressed on the cell surface by the progeny of T_N cells, but it is mostly lost by T_{CM} , and T_{EM} remains negative (Fig. 3b). Interestingly, MACSiBeads- and TransAct-stimulated T_N cells have higher frequency of $CCR7^+MART-1^+CD8^+$ (57.22 ± 6.8 % and 58.26 ± 10.1 %, respectively) when compared to $\alpha CD3 + \alpha CD28$ stimulation (30.5 ± 8.4 %). These data indicate that the phenotype and in particular the critical homing receptor expression can be modulated by the stimulation system.

To evaluate the homogeneity of transduced T cell subsets, we reanalyzed CD27 expression after transduction and expansion (Fig. 4a). Despite relatively consistent expression within each subset prior to culture, after 13–15 days, there was extensive patient-to-patient variability, with no significant differences between T_N , T_{CM} and T_{EM} , or between stimulation methods. We also checked beta-1, 3-glucuronyltransferase-1 (B3GAT1) encoding CD57 expression. In humans, CD57 is found on the surfaces of senescent effector cells induced by chronic infection [14]. The percentage of CD57-expressing cells progressively increases according to the degree of differentiation of the starting cell population after transduction with $T_N < T_{CM} < T_{EM}$ (Fig. 4b). It is possible to appreciate that in $\alpha CD3 + \alpha CD28$ -stimulated cells, all subsets showed similar $CD57^+$ expression. In contrast, bead-stimulated cells show differences in the CD57 expression among subsets, like the subsets analyzed ex vivo. Similarly, we tested the expression of LAMP-1 on the surface of the cells. LAMP-1 (CD107a), transiently located on the plasma membrane, can be used as a marker for $CD8^+$ T cell degranulation following stimulation. In Fig. 4c, we observe the percentage of $CD107a^+$ cells is similar in all subsets stimulated with soluble $\alpha CD3$ and $\alpha CD28$ (T_N : 27.6 ± 4.9 %; T_{CM} : 34.2 ± 1.9 %; T_{EM} : 32.3 ± 3.7 %), while both

MACSiBead- and TransAct-stimulated T_N cells had lower CD107a⁺ (MACSiBeads: 19.9 ± 3.2 %; TransAct: 22.8 ± 3 %) compared to T_{CM} (MACSiBeads: 33.5 ± 3.8 %; TransAct: 40.6 ± 2.4 %) and T_{EM} (MACSiBeads: 30.9 ± 3.3 %; TransAct: 46.4 ± 7 %) upon stimulation with MART1 expressing melanoma cells. Cytotoxic assay was also performed on transduced cells showing their capability to recognize and kill specifically the target cells but with no statistical differences among subsets and among the different stimulations (Online Source 3). All these results show that T cell subsets can be efficiently transduced upon stimulation and proliferate well for at least 2 weeks. As anticipated, the starting T cell population and, interestingly, the type of initial stimulation influenced the expression of markers that are known to correlate with the behavior of cells in vivo.

Functional characterization of the MART-1 TCR in T cells subsets

We next investigated the functional activity of the introduced MART-1 TCR and the differentiation status of transduced cells looking at cytokine production upon restimulation with a MART-1⁺HLA-A2⁺ melanoma cell line. Focusing on IL2 (Fig. 5a, b), we observed that a higher percentage of T_N CD8⁺ T cells produced IL2 when they were stimulated with MACSiBeads (13 ± 4.2 %) or TransAct (7.3 ± 1.3 %) compared to immobilized α CD3 + α CD28 (3.8 ± 0.7 %) stimulation. A similar effect was observed for TNF α -producing cells detected in the T_N subset when stimulated with MACSiBeads (42.2 ± 4.3 %) compared to immobilized α CD3 + α CD28 (22 ± 5.6 %) stimulation (Fig. 5c). In contrast, the method of stimulation did not influence the percentage of MART-1tet⁺ IFN γ ⁺ cells, and all three stimulation methods showed a trend to higher levels of IFN γ + cells in T_{CM} and T_{EM} CD8⁺ T cell subsets compare to T_N (though this was not statistically significant, Fig. 5d). Taken together, these results indicate TN-derived cells stimulated with MACSiBeads or TransAct showed an increased proportion of IL-2⁺ cells, and an equivalent or improved proportion that was TNF α . In contrast, the T_{CM} and T_{EM} CD8⁺ T cell subsets showed an increased proportion of IFN γ ⁺ cells. These results support a model of increased progress toward terminal differentiation from $T_N \rightarrow T_{CM} \rightarrow T_{EM}$.

Discussion

Genetic modification of T cells to engineer improved antitumor effects is an attractive strategy in many settings [28]. However, complete and durable tumor regression must be improved [29], and the use of selected T cells subsets as targets for genetic modifications is an attractive strategy toward this goal. Mouse models as well as clinical trials indicate that intrinsic properties related to the differentiation state of the adoptively transferred T cell populations are crucial to the success of ACT-based approaches [14, 30, 31]. In terms of efficacy in ACT for cancer, naive T cells have been shown to be more effective than memory T cells [16, 31, 32] and, within memory pools, T_{CM} cells show increased antitumor activity compared with T_{EM} cells in mice [33, 34], non-human primates [17] and humanized mouse models [35]. In addition, new evidence indicates that progeny cells derived from T_{CM} cells might persist and function in humans because of the reacquisition of CD62L marker [36]. These data suggest that in fact the quality rather than the quantity of transferred T cells determines their therapeutic activity and that fewer T cells which have been appropriately

selected and which require only minimal in vitro manipulation can be used for future therapies.

However, the translation of this principle to the clinic requires the development of GMP-compliant, clinical-scale techniques. In fact, the limited availability of GMP reagents for complex cell sorting and in vitro manipulation procedures is often a major roadblock for improving cellular therapies, which is often neglected by basic researchers. Rational development of GMP procedures and reagents may also lead to unexpected outcomes. For instance, Hinricks et al. found superior in vitro transduction and expansion of naïve human lymphocytes with non-GMP-compliant reagents and procedures. In contrast, we found all PBL subsets to have similar transduction efficiencies and similar in vitro expansions, when GMP-compliant processes and reagents were used.

Therefore, to directly assess the in vivo persistence and therapeutic capacity of these two subsets in a clinical trial, protocols for their parallel clinical-scale isolation and manipulation are required, relying on the use of GMP-grade materials and convenient and reliable processing steps. In the present manuscript, we report two GMP-compatible protocols for the enrichment of CD8⁺ naïve and central memory T cells before genetic modification for clinical use.

Employing the clinical-scale selection protocol, we achieve mean purities of 75.2 ± 15.5 and 84 ± 9.6 % (among CD8⁺ cells) for the T_N and T_{CM} subset, respectively. The identity of the subsets was confirmed further by phenotypic characterization using CCR7, CD127, CD27 and GrnzaA. The cell recovery for the various steps range from 70 to 90 % and lead to an overall cell recovery of approximately 60 % in the case of central memory enrichment, and 40 % for the naïve T cell subset. The decreased recovery of the T_N subset is due to one additional enrichment step.

Despite some variability between patients, naïve and central memory CD8⁺ T cells represent about 4 % of the PBMC population. Thus, starting from 3×10^9 total PBMCs for each enrichment, at least 5×10^7 cells of the desired population can be collected to initiate the in vitro manipulation. The mean expansion during the viral transduction was about 128 ± 12.8 -fold depending on the subset and stimulation reagent, yielding cell doses of more than 5×10^9 within 2 weeks from patient leukapheresis. Including a single additional rapid expansion step [37, 38] would allow dose escalating studies up to or beyond a maximum of 1×10^{11} T cells infused into the patient for testing the safety and then the efficacy of this treatment.

The in vitro stimulation of isolated T cell subsets also requires the evaluation of possible stimulation protocols. In contrast to whole PBMC stimulation done with soluble αCD3 antibodies and which relies on cross-linking by accessory cells, isolated T cell subsets require GMPgrade αCD3/αCD28 reagents that induce sufficient crosslinking to generate appropriate T cell activation. This is typically achieved by coating the αCD3/αCD28 antibodies to the plastic surface of the cell culture dish or cell-sized particles. Both procedures are difficult to integrate into a reliable and convenient cell generation process. Antibody coating to plastic surfaces is difficult to standardize and control, and large particles have to be efficiently removed from the cell product before infusion into patients. In

addition, large particles cannot be sterilized by filtration and are therefore difficult to use in closed systems under GMP cell culture conditions. Besides, these stimulation procedures do not allow easy adaptation of the signaling strength or variation of costimulatory molecules to the requirements of the particular subset. Therefore, we compared these standard reagents (α CD3 + α CD28 and MAC-SiBeads) with a new class of soluble α CD3/ α CD28-coated nanomatrix, TransAct. The TransAct reagent can be sterile filtered and added to the cell culture at the appropriate concentration and ratios just as soluble antibodies. At the end of the processing, the reagent can easily be removed via centrifugation of the cells. We have shown that all three reagents induce cell activation in T cell subsets and lead a CD8⁺ T cell expansion that is comparable or better than PBMC stimulated with soluble α CD3 antibody. Furthermore, the activated T cell subsets were efficiently transduced (30–90 %) and expressed the MART-1-specific TCR. While some variability was observed in the transduction efficiency among patients and between T cell subsets in these experiments, the number of cells expressing the functional TCR routinely exceeded the cell numbers required for a clinical trial.

Interestingly, we observed that the method of T cell activation can have significant effects on the phenotype and function of cells after subsequent transduction and expansion. Cells activated with MACSiBeads or TransAct show a tendency to preserve CCR7 expression especially on T_N cells and CD62L on both T_{CM} and T_N cells compared to cells activated with immobilized α CD3 + α CD28. Both molecules are central for migration into the lymph node and may confer improved in vivo persistence [39, 40]. Also the proportion of IL-2⁺ cells was increased in T_N in MAC-SiBeads- and TransAct-stimulated cells. Further improvement of the phenotypic and functional features might be achieved by culturing cells, prior and after the transduction, in an optimized cytokine cocktails, such as IL-7, IL-15 or IL-21, or alternate costimulatory ligands such as 4-1BBL [41–43]. Overall, the TransAct reagent offers an effective, variable and easy-to-use stimulation system, which will significantly facilitate and improve T cell culture under GMP conditions in automated and/or closed systems.

The choice to deplete CD4⁺ T cells is due to the broad heterogeneity of CD4⁺ T cell subsets that may lead to strong variability including adverse effects once reintroduced into the patient. However, the potential to formulate gene-modified T cell products that contain defined proportions of CD8⁺ and CD4⁺ T cells from distinct subsets needs to be explored in the future since CD4⁺ T cells also have been shown to exhibit antitumor activity, e.g., by providing help for CD8⁺ T cells [44, 45]. In the future, peripheral blood subsets that include CD4⁺ subsets might be enriched and investigated for clinical use, especially for CARs or TCRs that mediate their antitumor effects through CD4⁺ T cell-dependent mechanisms.

In summary, we provide an efficient and easy protocol for the GMP isolation, transduction and expansion of naïve and central memory T cells from patient leukapheresis products. The use of a new class of T cell stimulation reagent (TransAct) significantly simplifies the activation and expansion of isolated T cell subsets in closed GMP cell culture systems. The protocols developed here will form the basis for clinical trials using manipulated T cell subsets to treat cancer patients and will also allow the direct comparison of cellular products

derived from distinct T cell subsets, with the goal to improve T cell-based therapies via identification of critical cellular parameters for clinical efficacy and safety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a Naïve selection **b Central memory selection** **Fig. 1.**

Enrichment of CD8⁺ naïve and central memory T cell subsets. Freshly isolated PBMC from leukapheresis of melanoma patients have been used for the selection. Representative *dot plot* analysis from patient 4 for **a** naïve and **b** central memory CD8⁺ T cells. PBMC were stained at each selection step with CD4 and CD8 mAbs and among CD8⁺ T cells analyzed for the expression of CD45RO, CD45RA and CD62L markers

Fig. 2. The identity of the enriched subsets is confirmed by their phenotypic characterization. Enriched T cell subsets (T_N , T_{CM} , T_{EM}) and PBMC have been stained for CCR7, CD127, CD27 and GranzymeA expression. **a** Representative *dot plots* of CD8⁺ cells from patient 4 are shown, and reported numbers represent percentage of cells in the gate. The overall frequencies of **b** CCR7⁺; **c** CD127⁺; **d** CD27⁺ and **e** GranzA⁺CD8⁺ cells from 7 patients is shown

Fig. 3.

Higher percentage of bead-stimulated T_N CD8⁺ cells retains CD62L and CCR7 expression after expansion. PBMC were freshly isolated from leukapheresis of melanoma patients. CD8⁺ T cell subsets were enriched and then stimulated with αCD3 + αCD28 or MACSiBeads or TransAct in the presence of IL2. PBMC were stimulated with soluble αCD3 and IL2. Cells were transduced to express MART-1 TCR 60 h after stimulation. The data here were obtained 13–15 days after stimulation of each culture. Frequencies of **a** MART-1 tetramer⁺CD62L⁺ and **b** MART-1 tetramer⁺CCR7⁺ cells among CD8⁺ T cells are represented

Fig. 4.

Naïve and central memory cells stimulated with beads show less terminally differentiated phenotype. Freshly isolated T cell subsets and PBMC were stimulated as reported before. 60 h after stimulation, cells were transduced to express MART-1 TCR and cultured for a total of 13–15 days. After IL2 withdrawal, cells were stained for CD127, CD57 and CD27 markers or stimulated with MART-1⁺ HLA-A2⁺ melanoma cell line in the presence of CD107a antibody and Monensin for 5 h. Statistical analysis of **a** MART-1tetramer⁺ CD27⁺; **b** MART-1tetramer⁺ CD57⁺ and **c** CD107a⁺ cells among CD8⁺ T cells frequencies

Fig. 5.

Cytokine secretion upon MART-1 restimulation in CD8⁺ T cell subsets. Transduced T cell subsets and PBMC were cultured for a total of 13–15 days. Afterward cells were washed out from IL2 and rested for further 2 days and then restimulated with MART-1⁺ HLA-A2⁺ melanoma cell line for 6 h. The cytokine production was determined by intracellular staining. **a** *Dot plots* analysis show IL2 expression among CD8⁺ cells in T_N cell subsets stimulated with α CD3 + α CD28 or MACSiBeads or TransAct. *Graphs* show the frequencies

of of **b** IL2⁺; **c** IFN γ ⁺; and **d** TNF α ⁺ CD8⁺ T cells normalized for the percentage of MART-1⁺CD8⁺ cells in that subset

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Table 1
Cell recovery and population purity in naïve and central memory CD8⁺ T cell enrichments

Naïve enrichment		Central memory enrichment			
Representation of CD8 ⁺ CCR7 ⁺ CD45RA ⁺ cells Among PBMC		4.9 ± 3.8 %	Representation of CD8 ⁺ CD62L ⁺ CD45RA ⁻ cells Among PBMC		4.0 ± 1.3 %
Overall cell recovery After enrichment		44.6 ± 16.7 %	Overall cell recovery After enrichment		60.9 ± 23.8 %
Enrichment step	Purity (%)	Cell recovery (%)	Enrichment step	Purity (%)	Cell recovery (%)
CD4 depletion (among viable cells)	99.9 ± 0.02	72 ± 26	CD45RA depletion (among CD8 ⁺ T cells)	98.5 ± 1	71.2 ± 15.7
CD62L enrichment (among CD8 ⁺ T cells)	86.4 ± 7.8	77.5 ± 7.8	CD62L enrichment (among CD8 ⁺ T cells)	84 ± 9.6	88 ± 9.9
CD45RA enrichment (among CD8 ⁺ T cells)	75.2 ± 15.5	74.7 ± 21.5			

We determined the purity and the representation of the indicated populations via cytofluorimetric analysis. The yield of each population was calculated by multiplying the absolute cell number for the fraction of interest times the proportion of the same cells present in the same fraction. For Naïve enrichment, the absolute CD4⁺ T cell (among viable cells; CD4 depletion step), CD62L⁺CD8⁺ T cell (in the CD62L enrichment) and CD45RA⁺CCR7⁺CD8⁺ T cell (CD45RA enrichment) numbers were calculated. For the central memory enrichment, the absolute number of CD45RA⁺CD8⁺ (CD45RA depletion) and CD62L⁺CD45RA⁻CD8⁺ (CD62L enrichment) T cells was determined. The absolute number of each population was calculated in the starting fraction (before the selection step) and in the target fraction (after the selection step). The cell recovery in case of a depletion procedure was determined as: (Total cell number in the target fraction – absolute cell number of the indicated population in the target fraction) divided by (Total cell number of the starting fraction – absolute number of the indicated population, before the depletion) × 100 (%). For the enrichment steps, the cell recovery was calculated dividing the absolute number of the population of interest in the target by the absolute number of the same population in the starting fraction × 100 (%). The overall cell recovery was calculated dividing the number of CD8⁺CCR7⁺CD45RA⁺ (for the naïve enrichment) or CD8⁺CD45RA⁻CD62L⁺ (for the central memory procedure) obtained at the end of the selection by the absolute number of the same population in the starting fraction (before any manipulation). The data are the average ± standard deviation of 7 experiments