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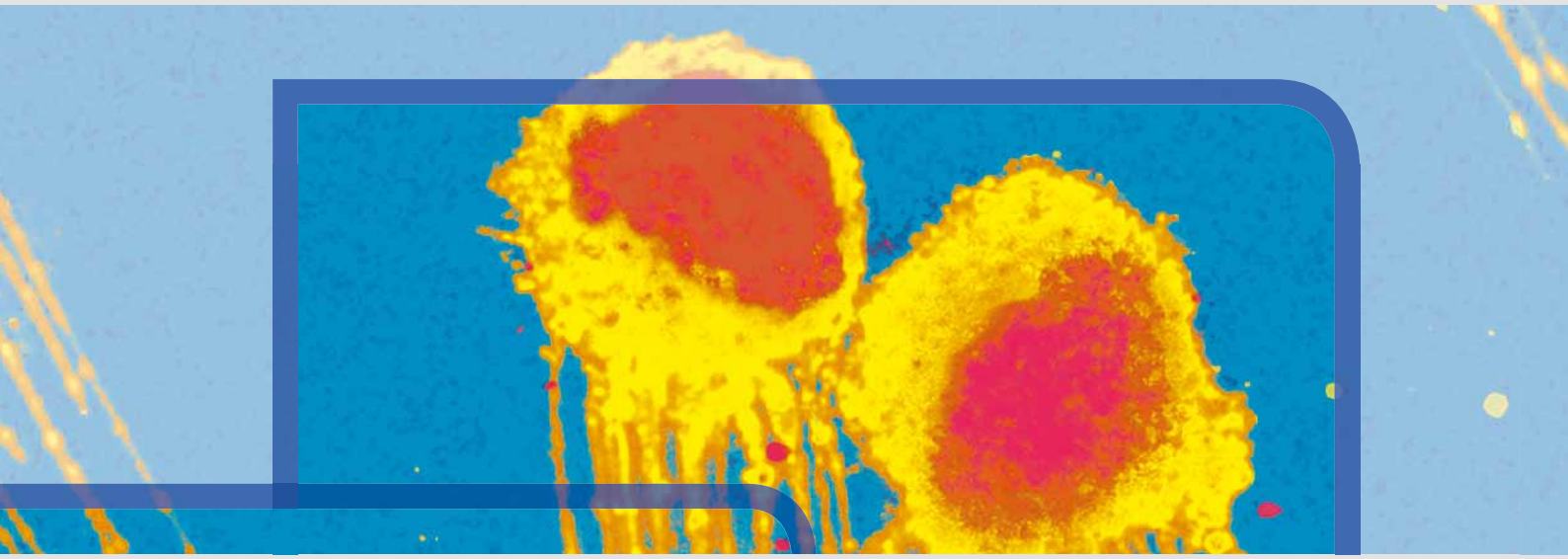

Enrichment, stimulation, and viral transduction of naive and central memory CD8+ T cells under GMP conditions for translational research towards the development of adoptive cell therapy of cancer patients

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Introduction

Adoptive immunotherapy involving the transfer of genetically engineered T cells can mediate tumor regression in patients with metastatic melanoma or other forms of cancer.[1-4] However, the efficacy of T cell immunotherapy depends on a number of critical steps. Autologous T cells collected from the patients are activated, genetically modified, expanded, and finally infused back into the patient to unfold their anti-tumor activity.

Peripheral blood T cells are heterogeneous with regard to their function and differentiation states, i.e., their self-renewal capacity and persistence. In particular terminally differentiated effector memory T (TEM) cells show poor persistence and only weak capacity to mediate tumor regression[1-5]. It has been shown that engineering of T cells can improve their anti-tumor reactivity and *in vivo* persistence.[6,7] Moreover, naive T cells (TN) show a higher efficacy in adoptive cell transfer in a cancer setting than memory T cells.[8-10] Among memory T cells, central memory T cells (TCM) were shown to exert a higher anti-tumor activity than TEM cells in mice[11,12], non-human primate[13], and humanized mouse models[14]. However, the translation of this principle into therapeutic strategies is hampered by the lack of appropriate protocols for the clinical-scale isolation of CD8+ TN and TCM cells, as well as GMP-compliant reagents and reliable processes for cell activation and transduction.

One of the current standard procedures for cell activation involves soluble CD3 antibodies in combination with, for example, autologous antigen-presenting cells (APCs) for cross-linking. This protocol is highly dependent on the quality of the cross-linking cells and requires large amounts of viral supernatants, which is a major cost factor in this process. The other standard T cell activation process involves CD3 and CD28 antibodies immobilized on solid surfaces, such as cell culture vessels or large cell-sized particles.[8,13] These methods have major drawbacks, as they are time-consuming and difficult to standardize for GMP compliance. Moreover, large particles need to be removed prior to infusion of T cells into the patient.

We developed a reliable method for the enrichment of CD8+ TN and TCM cells under GMP conditions. We also introduced a new flexible nanomatrix conjugated with CD3 and CD28 antibodies (TransAct™ Reagent), which allows easier handling for the activation of T cells in a closed system under GMP conditions. The activated TN and TCM cells isolated from blood of melanoma patients were expanded and transduced efficiently. TN and TCM cells activated with TransAct Reagent or CD3/CD28-conjugated cell-sized MACSiBead™ Particles showed only minimal effector differentiation.[15]

Materials and methods

Enrichment of human CD8+ TN and TCM cells

For all experiments fresh leukapheresis samples from patients with metastatic melanoma were used. Patients were enrolled in clinical trials approved by the National Cancer Institute Institutional Review Board. PBMCs were prepared by density gradient centrifugation (Ficoll™ Hypaque™) and washed twice in PBS. For each enrichment, 2–4×10^9 PBMCs were used.

TN cells were enriched with a three-step procedure. Firstly, PBMCs were incubated with CliniMACS® CD4 MicroBeads on a rotator for 30 min at room temperature (RT). Cells were washed by centrifuging and resuspending in 100 mL of buffer A, i.e., CliniMACS PBS/EDTA Buffer supplemented with 0.5% human albumin (Plasbumin®-25 25%, USP; Talecris Biotherapeutics), and transferred to a collection bag. Magnetically labeled CD4+ cells were depleted using the CliniMACS Plus Instrument (program Depletion 2.1) and the CliniMACS Tubing Set LS. Secondly, the unlabeled target cells were incubated with CliniMACS CD62L MicroBeads for 15 min in cold buffer A and washed. Target cells were enriched using the CliniMACS Plus Instrument (program Enrichment 3.2) and the CliniMACS Tubing Set. Finally, the target fraction was labeled with a CD45RA-Biotin, human antibody and Anti-

Biotin MACSiBead Particles. Cells were further enriched using the MACSiMAG™ Separator.[15] TCM cells were enriched with a two-step procedure. PBMCs were incubated with CliniMACS CD4 MicroBeads and CliniMACS CD45RA MicroBeads (in cold buffer A) on a rotator for 30 min at RT. Subsequently, the cells were washed, resuspended in 100 mL of buffer A, and transferred to a collection bag. Magnetically labeled cells were depleted using the CliniMACS Plus Instrument (program Depletion 2.1) and the CliniMACS Tubing Set LS. The unlabeled target cells were incubated with CliniMACS CD62L MicroBeads for 15 min in cold buffer A and washed. Target cells were enriched using the CliniMACS Plus Instrument (program Enrichment 3.2) and the CliniMACS Tubing Set.[15]

Flexible nanomatrix for T cell stimulation (TransAct Reagent)

The TransAct Reagent was provided by Miltenyi Biotec. TransAct Reagent 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 µg/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

Three different methods were used for stimulation of the enriched T cell subsets: 1) CD3 antibody coated on the cell culture dish (OKT3; 10 µg/mL) in combination with soluble CD28 antibody (15E8; 1 µg/mL), referred to as CD3/CD28, 2) MACSiBead Particles loaded with CD3, CD28, and CD2 antibodies, and 3) TransAct Reagent. Interleukin 2 (IL-2; 300 IU/mL) was present in the cell culture for all three approaches.[15] T cells were transduced using the mF5 retrovirus encoding an anti-MART-1 T cell receptor (TCR) comprising constant regions of the mouse TCR α and β chains in addition to human TCR CDR antigen-combining regions[16]. Transductions were performed as described.[17]

Flow cytometry

To analyze the phenotypes of the T cell subsets, cells were labeled with fluorochrome-conjugated antibodies against CD8, CD4, CD45RA, CD62L, CD127, IFN-γ, IL-2, 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). For the analysis of intracellular cytokine production, cells were cocultured with the 624 (MART-1$^+$HLA-A2$^+$) melanoma cell line in the presence of brefeldin A. After 6 hours cells were labeled with the CD8 antibody, fixed, and permeabilized with the Inside Stain Kit (Miltenyi Biotec). Subsequently, cells were analyzed by flow cytometry.

Statistical analysis

Groups were compared using the two-tailed unpaired t-test. The error bars in the graphs represent the standard error of means.

Results

Clinical-scale enrichment of human CD8$^+$ TN and TCM cells

Human CD8$^+$ T cells represent a heterogeneous population of TN, TCM, and TEM cells, which can be distinguished by their expression of CD45RA and CD62L[18] (table 1). We used these phenotypic characteristics to isolate TN and TCM cells. TN cells were enriched in a three-step procedure, involving CD4$^+$ cell depletion, positive selection of CD62L$^+$ cells, and a second positive selection of CD45RA$^+$ cells. Purity and recovery are shown in table 1. The remaining CD8$^-$ cells contained in the target fraction were mainly NK and B cells.

TCM cells were enriched in a two-step procedure, i.e., simultaneous depletion of CD4$^+$ and CD45RA$^+$ cells and subsequent positive selection of CD62L$^+$ cells. The unlabeled non-target fraction from the CD62L$^+$ cell enrichment step was enriched in CD8$^+$CD45RA$^-$CD62L$^-$ cells and represented the TEM cells in our *in vitro* experiments.

Phenotypic characterization of CD8$^+$ TN and TM cells

The CD8$^+$ cell subsets are characterized by differential expression of a number of additional markers. As CD62L and CCR7 are generally co-expressed in TN and TCM cells, we analyzed the enriched cell populations for CCR7 surface expression (fig. 1) and found that 70±5.5% of the TN cells and 54±7% of the TCM cells expressed CCR7. In contrast, only 13.2±2.4% of the TEM cell fraction expressed CCR7 (see fig. 1 for representative dot plots). A large proportion of the TN cell fraction (62.5±5.7%) expressed CD127 (IL-7R), whereas expression in TCM and TEM cells amounted to only 30±3.7% and 30.7±7.3%, respectively. Expression of CD27 is known to positively correlate with replicative capacity and tumor response[19,20]. We found that CD27 expression was significantly higher in TN and TCM cells than in TEM cells (88±1.8% and 80.5±5.3 vs. 62±5.4%). In contrast, granzyme A expression was higher in TEM cells (85±2.2%) compared to TN and TCM cells (16.7±3.6% and 42.5±6.6%). This is an agreement with the notion that lymph node homing capacity and the acquisition of effector function are coordinately regulated[18,21]. Taken together, these data indicate that the GMP-compliant procedures used for enrichment of TN and TCM cells yield populations with distinct, typical phenotypes (fig. 1).

Transduction of T cell subsets with antigen-specific TCR

The standard protocols for *in vitro* activation of T cells involve autologous antigen-presenting cells (APCs), which are required for cross-linking of soluble CD3 antibodies

T cell subset	Phenotype	Purity	Recovery
Naive T (TN) cells	CD8$^+$CD45RA$^+$CD62L$^+$	75.2±15.5%	44.6±16.7%
Central memory T (TCM) cells	CD8$^+$CD45RA$^-$CD62L$^+$	84±9.6%	60.9±23.8%
Effector memory T (TEM) cells	CD8$^+$CD45RA$^-$CD62L$^-$	n.d.	n.d.

Table 1 Phenotypes of human CD8$^+$ T cell subsets. Purities (among CD8$^+$ cells) and recoveries after enrichment with the CliniMACS System are shown. Numbers indicate means ± SEM (n=6). Data were adapted from reference 15.

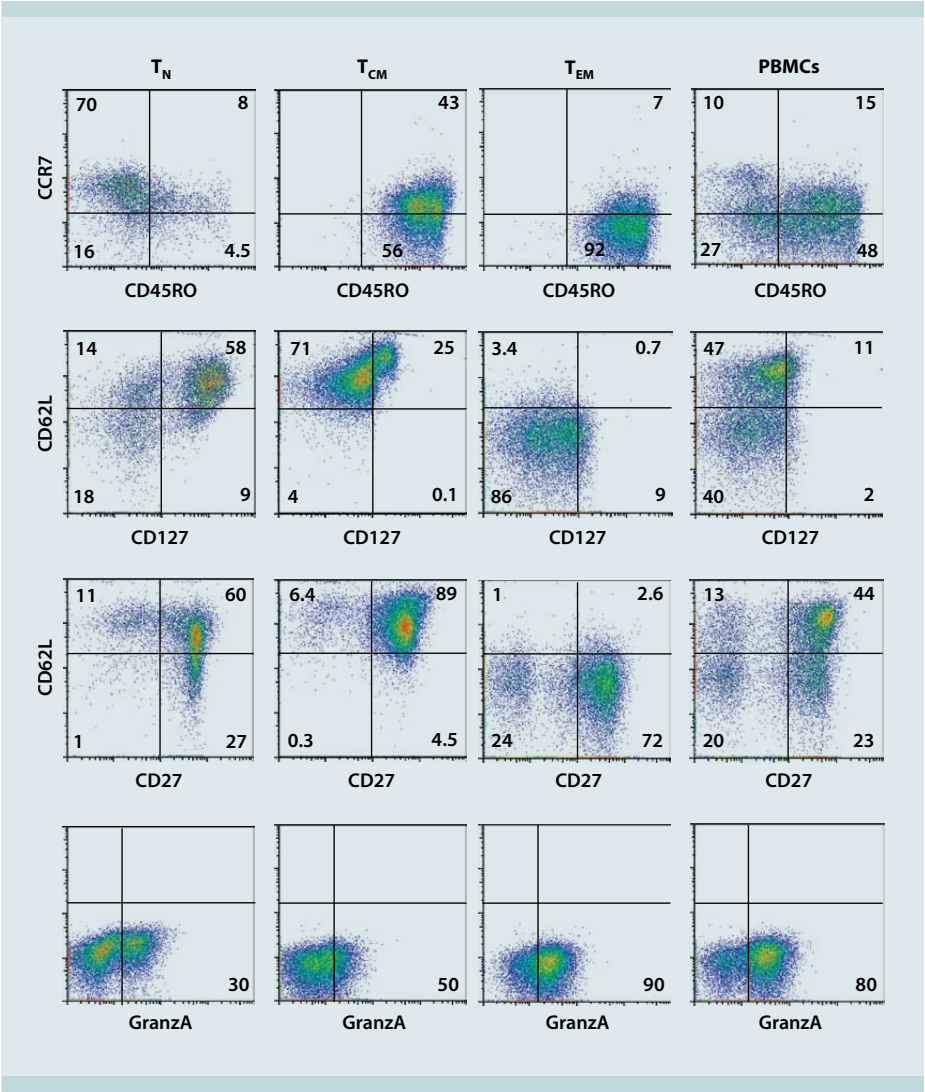

Figure 1 Enriched TN, TCM, and TEM cells show distinct phenotypes. Enriched T cell subsets and PBMCs were analyzed for expression of CCR7, CD45RO, CD62L, CD127, CD27, and granzyme A (GranzA). Representative dot plots are shown. Numbers indicate the percentages of cells in the respective gates. *0.01 < P < 0.05; **0.005 < P < 0.01; ***P < 0.005. Data were adapted from reference 15.

and costimulatory signals. Since APCs are removed during enrichment of the T cell subsets, an alternative approach was required to effectively stimulate the enriched T cells. We tested three methods: 1) CD3 antibodies coated onto the cell culture vessel plus soluble CD28 antibody, 2) CD3 and CD28 antibodies linked to MACSiBead Particles, and 3) TransAct Reagent, a novel GMP-compatible reagent, which consists of CD3 and CD28 antibodies immobilized on a novel flexible nanomatrix. In contrast to MACSiBead Particles, the TransAct Reagent can be effectively removed from the culture by centrifugation. Thus, the reagent can be handled similarly to soluble antibodies.

$CD8^+$ TN, TCM, and TEM cells were stimulated for 60 hours and then transduced with a retroviral vector encoding the anti-MART-1 TCR. Ten days after transduction, the transduction efficiency was similar for all three T cell subsets, regardless of the stimulation protocol, as determined by MART-1 tetramer staining (data not shown). We analyzed the transduced cell subsets for expression of CCR7 and CD62L after 13–15 days of expansion. CD62L was expressed at equally high levels on TN and TCM cells, whereas TEM cells showed a lower cell surface expression of this marker (fig. 2). CCR7 was highly expressed on TN cells, in contrast to TCM cells, which showed reduced surface expression, and TEM cells, which were CCR7 negative. TN cells stimulated with TransAct Reagent or MACSiBead Particles showed a higher frequency of $CCR7^+$MART-1 $tetramer^+CD8^+$ cells (57.22±6.8% and 58.26±10.1%) compared to cells stimulated with CD3/CD28 (30.5±8.4%). This indicates that the phenotype, in particular expression of

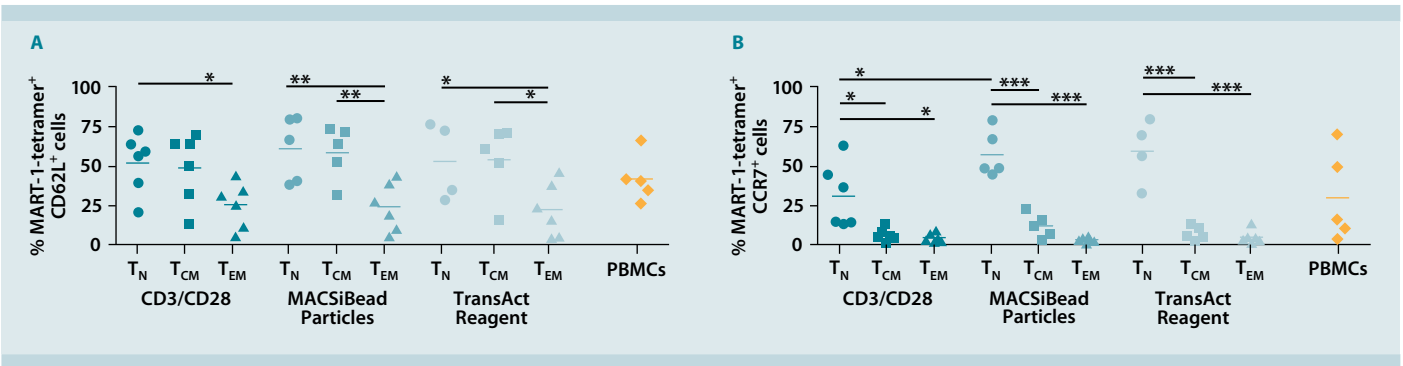

Figure 2 Expression of CD62L and CCR7 in MART-1 $tetramer^+$ TN, TCM, and TEM cells. The three T cell subsets were enriched from PBMCs and stimulated with CD3/CD28 antibodies, MACSiBead Particles, or TransAct Reagent in the presence of IL-2. PBMCs were stimulated with soluble CD3 and CD28 antibodies. Cells were transduced 60 hours after stimulation to express MART-1-specific TCR. Cells were analyzed by flow cytometry 13–15 days after stimulation. Data indicate frequencies of (A) MART-1 $tetramer^+CD62L^+$ cells and (B) MART-1 $tetramer^+CCR7^+$ cells among $CD8^+$ cells. Data were adapted from reference 15.

the homing receptor CCR7, can be modulated by the method of cell stimulation.

Taken together, our results show that the enriched T cell subsets can be efficiently stimulated, transduced, and expanded over two weeks. However, expression patterns in expanded cells can differ from the initial cell population, and marker expression varies dependent on the stimulation method, which may have an effect on the cells' behavior *in vivo*.

Functional analysis of T cell subsets

To test whether the MART-1 TCR in transduced cells was functional, we re-stimulated the T cell subsets with a MART-1$^+$HLA-A2$^+$ cell line and analyzed the production of IL-2, TNF-α, and IFN-γ. The percentage of CD8$^+$IL-2$^+$ TN cells was higher when they had been stimulated with MACSiBead Particles (13±4.2%) or TransAct Reagent (7.3±1.3%) compared to CD3/CD28 (3.8±0.7%) (fig. 3B). Similarly, TN cells stimulated with MACSiBead Particles showed a higher percentage of TNF-α-producing cells (42.2±4.2%) than CD3/CD28-stimulated TN cells (22±5.6%) (fig. 3D). The percentage of IFN-γ-producing T cells was similar for all three methods of stimulation (fig. 3C). There was a slight trend towards increased percentages of IFN-γ$^+$ cells in TCM and TEM cells in comparison to TN cells.

Compared to CD3/CD28-stimulated TN cells, the cells stimulated with MACSiBead Particles or TransAct Reagent showed an increase in IL-2, equivalent or increased TNF-α levels, and equivalent or reduced IFN-γ levels, which indicates less progress towards terminal differentiation.

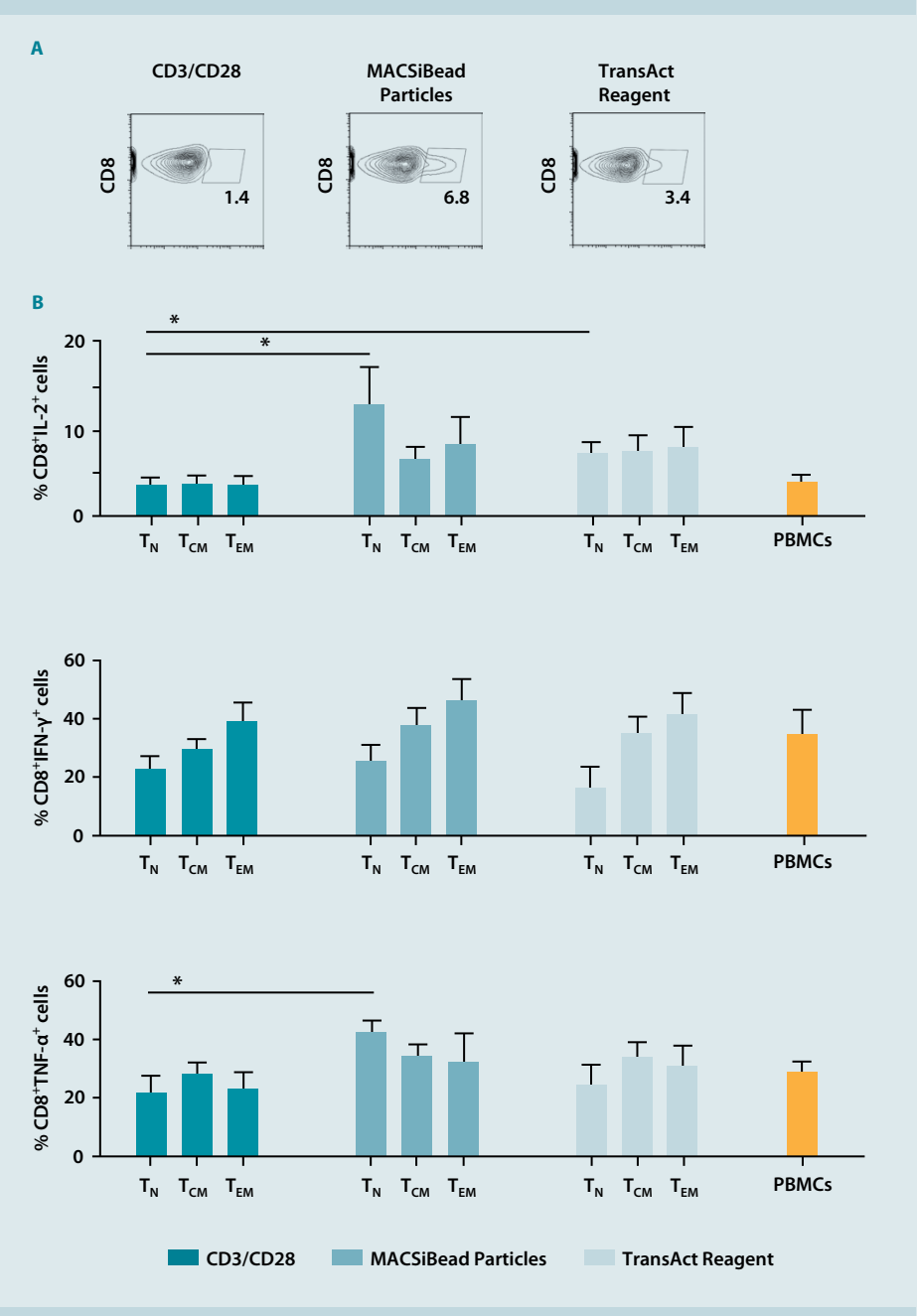

Figure 3 Cytokine production by TN, TCM, and TEM cells following re-stimulation with MART-1$^+$ melanoma cells. T cells were stimulated using the different protocols as indicated, and transduced to express a MART-1-specific TCR. Transduced T cell subsets and PBMCs were cultured for a total of 13–15 days and then washed to remove IL-2. Cells were cultured for two more days and re-stimulated with a MART-1$^+$HLA-A2$^+$ melanoma cell line for 6 hours. Cytokine production was determined by intracellular staining. (**A**) IL-2 expression in CD8$^+$ TN cells. Numbers indicate percentages of IL-2$^+$ cells among CD8$^+$ cells. (**B**) Frequencies of CD8$^+$IL-2$^+$, CD8$^+$IFN-γ$^+$, and CD8$^+$TNF-α$^+$ cells among CD8$^+$MART-1 tetramer$^+$ cells. *0.01 < P < 0.05; **0.005 < P < 0.01; ***P < 0.005. Data were adapted from reference 15.

Conclusion

- We developed a reliable and easy-to-perform GMP-compliant method for the enrichment of CD8$^+$ TN and TCM cells from leukapheresis products.
- We developed a novel reagent for effective T cell stimulation, TransAct Reagent, which can be sterile filtered and added to cell cultures at defined concentrations. Excess reagent can be easily removed from the cells by centrifugation.
- The enriched T cell subsets can be stimulated and efficiently transduced to produce MART-1-specific cells.
- Following stimulation of T cells with TransAct Reagent or MACSiBead Particles, expression of CD62L and CCR7 is preserved, in particular on TN cells, which may confer improved *in vivo* persistence to the cells.
- The enrichment and expansion procedure yields a high number of cells providing a solid basis for future translation into clinical studies.

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