Low interleukin-2 concentration favors generation of early memory T cells over effector phenotypes during chimeric antigen receptor T-cell expansion

TANJA KAARTINEN¹, ANNU LUOSTARINEN¹, PILVI MALINIEMI¹,², JONI KETO², MIKKO ARVAS³, HEINI BELT³, JONNA KOPONEN³, ANGELICA LOSKOG¹, SATU MUSTJOKI⁵,⁶, KIMMO PORKKA⁵, SEppo YLÄ-HERTTUALA³,⁷ & MATTI KORHONEN¹

¹Advanced Cell Therapy Centre, Finnish Red Cross Blood Service, Helsinki, Finland, ²Research & Development, Finnish Red Cross Blood Service, Helsinki, Finland, ³Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland, ⁴Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden, ⁵Hematology Research Unit Helsinki, Biomedicum Helsinki, Department of Medicine, Division of Hematology, University of Helsinki, Helsinki, Finland, ⁶Department of Clinical Chemistry and Hematology, University of Helsinki, Helsinki, Finland, and ⁷Heart Center, Kuopio University Hospital, Kuopio, Finland

Abstract

Background. Adoptive T-cell therapy offers new options for cancer treatment. Clinical results suggest that T-cell persistence, depending on T-cell memory, improves efficacy. The use of interleukin (IL)-2 for in vitro T-cell expansion is not straightforward because it drives effector T-cell differentiation but does not promote the formation of T-cell memory. We have developed a cost-effective expansion protocol for chimeric antigen receptor (CAR) T cells with an early memory phenotype.

Methods. Lymphocytes were transduced with third-generation lentiviral vectors and expanded using CD3/CD28 microbeads. The effects of altering the IL-2 supplementation (0–300 IU/mL) and length of expansion (10–20 days) on the phenotype of the T-cell products were analyzed.

Results. High IL-2 levels led to a decrease in overall generation of early memory T cells by both decreasing central memory T cells and augmenting effectors. T memory stem cells (TSCM, CD95⁺CD45RO⁻CD45RA⁺CD27⁺) were present variably during T-cell expansion. However, their presence was not IL-2 dependent but was linked to expansion kinetics. CD19-CAR T cells generated in these conditions displayed in vitro antileukemic activity. In summary, production of CAR T cells without any cytokine supplementation yielded the highest proportion of early memory T cells, provided a 10-fold cell expansion and the cells were functionally potent.

Discussion. The number of early memory T cells in a T-cell preparation can be increased by simply reducing the amount of IL-2 and limiting the length of T-cell expansion, providing cells with potentially higher in vivo performance. These findings are significant for robust and cost-effective T-cell manufacturing.

Key Words: chimeric antigen receptor T cells, effector function, human, interleukin-2, T-cell expansion, T-cell memory

Introduction

Adoptive T-cell therapy, the administration of ex vivo processed T cells, provides new treatment options for refractory or advanced cancer [1]. Promising initial clinical results demonstrated in melanoma with tumor-infiltrating lymphocytes (TILs) [2] are now being followed by genetically engineered T cells. Use of T-cell receptor (TCR)–redirected T cells, e.g., in myeloma targeting NY-ESO-1 [3], and especially, the remarkable clinical success of chimeric antigen receptor (CAR) T cells in B-cell malignancies [4], justify the enthusiasm.

Nevertheless, clinical experience has shown that determining the in vivo potency of T-cell products for cancer is not straightforward. The durable control of cancer is not the result of a single cell population or a sole direct mechanism such as tumor cell killing. The
durable control of cancer is rather due to a dynamic anti-tumor T-cell response entailing different cell types working in a temporally orchestrated manner during treatment and disease control. Furthermore, effector function, e.g., tumor cell killing, is connected to the short life expectancy on a per cell basis. Clinical results suggest that T-cell persistence improves therapeutic response [5,6] and this depends on telomere length [7] and development of immunologic memory [8–10].

Pre-clinical animal models have confirmed that memory T cells are pivotal for in vivo anti-tumor efficacy. Terminally differentiated effector T cells with the most potent cytotoxic functionality are not the optimal therapeutic in the in vivo setting; instead, pure preparations of less-differentiated memory T cells with greater in vivo proliferation and survival are more effective [11]. Thus, particular T-memory subsets with greater proliferative capacity should be pursued [11–14]: central memory T cells (T_CM) instead of effector memory (T_EM, both defined in Sallusto et al. [15]), or even the most primitive T memory stem cells (T_SC, defined in Gattinoni et al. [12]). T_SC provide not only superior potential for proliferation but also self-renewal.

Studies in cancer-targeting adoptive T-cell therapy have often focused solely on direct tumor cell killing potency [16]. However, there is increasing evidence for the importance of CD4+ cells in therapeutic T-cell products. CD4+ T cells may have several roles in cancer treatment provoking bystander immunity, supporting the activation of CD8+ cells as well as directly inhibiting tumor growth [17]. In the tumor milieu CD4+ cells have also shown to enhance the recruitment, proliferation and cytotoxic function of CD8+ cells [18]. Furthermore, in one clinical study T-cell persistence and clinical efficacy were associated with the presence of both less-differentiated T memory (T_M) cells as well as CD4+ T cells in the cell product [10]. Interestingly, CD4+ cells are indispensable for functional CD8+ memory formation in mice [19,20]. It is not known if the indicated CD4+ importance for clinical efficacy was linked to memory formation.

In vivo, T-cell proliferation typically only takes place during an immune response and thus T-cell activation, proliferation and effector/memory differentiation are tightly coupled [21]. In vitro T-cell expansion is achieved with signals mimicking normal T-cell activation [1]. Signaling through CD3 and CD28 in vivo provokes autonomous IL-2 production by the activated T cells, which is central to clonal expansion. Likewise, T-cell expansion in vitro is also characteristically boosted with interleukin (IL)-2, “the T-cell growth factor.” However, IL-2 drives T cells toward effector differentiation at the expense of memory cell formation [21]. Both IL-2 signaling strength and duration are known to affect CD8 memory T-cell development [22]. Furthermore, excessive stimulation during in vitro expansion may lead to cell exhaustion [23], yielding less potent T cells for therapy. Thus, generation of effective T-cell based cancer therapeutics requires a delicate balance between the final cell dose and the quality of the cells.

Currently, several approaches have been proposed for increasing the proportion of less-differentiated T cells. The replacement of IL-2 with IL-7, IL-15 and IL-21 uses the concept of physiological homeostatic T-cell proliferation [24]. This favors early memory T-cell generation from normal donors [25–27] as well as from cancer patient–derived cells [28] and improves in vivo anti-tumor functionality in pre-clinical models [11,29,30]. Clinical evidence is pending but preliminary results in four melanoma patients showed only limited persistence of GD2-targeted CART cells expanded using IL-7 and -15 [31]. Selection of more primitive starting T-cell populations prior to in vitro expansion [14] and use of signaling inhibitors that prevent effector differentiation [32] are other strategies for increasing the number of less-differentiated T cells for adoptive T-cell therapy.

Increasingly sophisticated cell manufacturing methods have been developed to achieve product reproducibility and high efficiency. Multistep manufacturing procedures may be more vulnerable and could hinder the reproducibility of the manufacturing method, perhaps leading to situations where the patient will not receive the needed cell therapy product. Each manufacturing step and production material included in the method increases substantially the labor needed to fulfill the Good Manufacturing Practice (GMP) requirements because all the raw materials used need to be controlled as well. This ultimately affects the overall production costs and the cost-effectiveness of the treatment.

In this work we investigated if T cells with preferred early memory phenotypes could be expanded without cytokine supplementation and extra manipulation. We have dissected the relative contributions of simple in vitro cell expansion conditions (IL-2 concentration, the length of expansion) to the cell composition of T-cell product and therapeutic potency. We pose that an optimal cancer-targeting T-cell product encompasses appropriate subpopulation heterogeneity, providing (i) rapid killing efficiency (activated CD8+ effectors), (ii) CD4+ T cells and (iii) a considerable proportion of less-differentiated, early memory T cells. In vitro functional studies were performed to assess the functional significance of these results in the context of adoptive T-cell therapy. In this study we have developed an effective and lean T-cell expansion protocol that can be applied to the production of clinically potent CAR T cells.
Materials and methods

T-cell expansion

Peripheral blood mononuclear cells (PBMC) from 10 healthy blood donors were isolated from buffy coats and from two leukemia patients from fresh whole blood samples by density gradient centrifugation using Ficoll-Paque Premium (GE Healthcare). Informed consent was obtained and the study and the use of human material were approved by the institutional review boards at the Finnish Red Cross Blood Service and the Hospital district of Helsinki and Uusimaa. Either fresh or cryopreserved cells were magnetically enriched for T cells and activated using Dynabeads Human T-Expander CD3/CD28 (Life Technologies) at a 3:1 bead to cell ratio. Cell expansion was conducted in small-scale cultures in X-VIVO 15 (Lonza) with 5% human AB serum (Gemini or Seralab) starting with a cell density of 1 × 10^6/mL. Expansions from individual donors were cultured in parallel under five different IL-2 (Proleukin, Novartis) concentrations (0, 5, 20, 100 and 300 IU/mL). From the third day onward, the cell density was adjusted to 0.5 × 10^6/mL with IL-2–supplemented culture media every 2–3 days.

Flow cytometry

Prior to phenotyping, the cells were fixed with paraformaldehyde. The following antibodies from BD Biosciences were used for staining: anti-CD3-fluorescein isothiocyanate (FITC), R-phycocerythrin (PE), BV510 (clone UCHT1) or FITC from Miltenyi Biotec (clone BW264/56), CD4-peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5) or BV510 (clone SK3), CD8-AlexaFluor647 or BV421 (clone RPA-T8), γδTCR-PE (clone 11F2), CD56-allophycocyanin (APC, clone B159), CCR7-PE (clone 150503), CD27-PerCP-Cy5.5 (clone M-T271), CD45RA-APC (clone HI100), CD45RO-BV421 (clone UCHL1) and CD95-PE (clone DX2). B-cell elimination from cell expansions was confirmed with anti-CD19-PE (clone HIB19). Appropriate isotype and fluorescence minus one (FMO) controls were used. Memory T-cell subtypes were identified according to the expression of CD27, CD45RA, CD45RO and CD95 as explained in Table I. Altogether 11 expansions (nine healthy donors, two patients) were phenotyped for memory T-cell status. Six of the expansions were transduced with CAR, three with green fluorescent protein (GFP) and two were T-cell expansions without transduction.

All flow cytometric analyses were performed using BD FACSAria IIu (BD Biosciences) and FlowJo (version 7.6.5 or 10, TreeStar) software.

Table I. Expression patterns used for T-cell subtype phenotyping.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>CD95–</th>
<th>CD45RO−</th>
<th>CD45RA−</th>
<th>CD27+</th>
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<tr>
<td>Naïve</td>
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<tr>
<td>TSCM</td>
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<tr>
<td>TSCM-abl</td>
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<td>TCM</td>
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<td>Tm</td>
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<tr>
<td>Tef</td>
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NOTE. Sequential differences in expression between subtypes are shown by underlining. TSCM, TSCM-abl and TCM cells represent less-differentiated, early memory T cells. Tef, effector T cell.

Construction of lentiviral vectors

The second-generation (2G) CD19-CAR (CD19-scFv/CD28/2) was edited from the third-generation (3G) construct (CD19-scFv/CD28/4-1BB/z [33]) by removing the 4-1BB sequence. Self-inactivating 3G lentiviral vectors (LVs) encoding CARs were generated by cloning the transgenes (GenScript) into LVs with human phosphoglycerate kinase promoters [34].

Lentiviral transduction and analysis of CD19-CAR expression

Two days after the activation, T cells were transduced with LVs encoding either the 2G or 3G CD19-CAR construct or GFP (data not shown) using a multiplicity of infection of 10. CD19-CAR-expressing cells were detected by flow cytometry using an AlexaFluor647-conjugated F(ab′)2 fragment goat anti-human immunoglobulin (Ig)G(H + L) (Jackson Immunoresearch, Inc.). CAR-positive cells were gated using untransduced or empty LV-transduced cells as controls. The background was eliminated by subtracting the percentage of positive cells in control samples from the percentage of positive cells in CD19-CAR-transduced samples. Control cells were cultured with 20 IU/mL IL-2.

B-cell lines

SupB-15 (CD19+ B-lineage acute lymphoblastic leukemia [ALL]) cell line was purchased from ATCC. NALM-6 (CD19+ B lineage ALL) was obtained from Dr O. Lohi (University of Tampere, Finland). Cell lines were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 100 IU/mL penicillin and 100 µg/mL streptomycin (Life Technologies).

Functional characterization of CART cells

CD19-CART-cell functionality was tested using CD19-expressing SupB-15 or NALM-6 B lineage ALL cells. Cell proliferation, degranulation and cytokine secretion assays were performed using cryopreserved and
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thawed cells, and fresh cells were used for cytotoxicity testing.

To measure proliferation in response to target cells, CD19-CAR T cells were labeled with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Life Technologies), and co-cultured for 4 days with NALM-6 at an effector-target cell ratio (E:T) of 10:1. CFSE dilution as a measure of proliferation was detected using flow cytometry.

Cytometric bead array (CBA) was used to measure the release of IL-2 and interferon (IFN)γ during a 1-day 1:1 co-culture of CD19-CAR T and NALM-6 cells. The cytokine concentrations were measured from the culture media using the BD CBA Human Soluble Protein Master Buffer Kit, CBA IL-2 Flex Set and CBA IFN-G Flex Set (BD Biosciences). Results were analyzed using FCAP Array Software Version 3.0 (BD Biosciences).

To measure degranulation in response to target cells, T cells were incubated at 1:1 with NALM-6 targets for 4 h in the presence of a CD107a antibody (BD Biosciences, clone H4A3). Degranulating T cells were identified by the surface expression of the lysosomal-associated protein CD107a using anti-CD107a-PE and flow cytometry.

Target cell killing efficacy was evaluated using the calcine release cytotoxicity assay. CD19-expressing SupB-15 or NALM-6 B-ALL target cells were labeled with calcine-acetoxymethyl (Life Technologies) and co-cultured at E:T ratios ranging from 0.3:1–30:1. After a 4-h incubation, target cell killing efficiency was determined by measuring the amount of fluorescent calcine released into the culture media using a FLUOstar Omega microplate reader (BMG Labtech).

Statistics

The differences between expansion conditions were analyzed with Mann-Whitney test and unpaired t test with two-sided P values. In analysis of CBA results, means of multiple groups were compared using one-way analysis of variance (ANOVA) and Tukey multiple comparisons test. Differences were considered statistically significant when P < 0.05. Analyses were performed using GraphPad Prism (version 6.07) software. The dependence of cell lysis percentage on cell ratios and IL-2 levels was modelled with a linear model using R (version 3.3.1 [35]; see Supplementary file for further details).

Results

T-cell expansion in low IL-2 concentration bolsters early memory T cells instead of effector phenotypes

IL-2 is a powerful cytokine inducing both clonal T-cell expansion and effector differentiation. We asked if the amount of exogenous IL-2 could be optimized for expansion of therapeutic T-cell preparations. T cells from peripheral blood of nine healthy donors and two patients with leukemia were activated using microbeads carrying CD3 and CD28 antibodies (Dynabeads) and cultured with varying concentrations of exogenously added IL-2 ranging from 0–300 IU/mL. T cells in most expansions were transduced to express either CAR (n = 6) or GFP (n = 3). On the tenth day of expansion, memory T-cell status was analyzed (Figure 1A). First, we analyzed the proportions of early TM cells, corresponding to TSCM, TSCM-like and TCM cells. Cultures without or with only 5 IU/mL IL-2 had an 88%–89% mean proportion of early TCM (Figure 1B). When higher IL-2 concentrations were used for T-cell expansion, the proportion of early TCM decreased, reaching 61% when using 300 IU/mL IL-2. The transduction process did not influence the memory T-cell composition as parallel untransduced control expansions gave similar results (data not shown). Thus, the increment of IL-2 decreased the proportion of early memory T cells.

Each T memory cell subtype, including TSCM, TSCM-like, TCM and TEM cells, as well as effector T cell (TEff), was present in the expanded products on day 10, whereas naïve T cells (CD95−) were absent after expansion. Increasing the amount of exogenously added IL-2 had its most profound effects on two particular memory T-cell subtypes: it decreased TCM and increased TEff levels (Figure 2). TCM decreased from the mean level of 32% accomplished without any exogenous IL-2 to around 10% when using 20 IU/mL or more. Conversely, very few TEff were detected in cultures with the two lowest IL-2 conditions (0 and 5 IU/mL), but their mean proportion increased with increasing exogenous IL-2, reaching 27% at the highest concentrations. The proportion of the most abundant T-cell subtype, the TSCM-like population comprising roughly half of the cells, was not substantially influenced by the amount of IL-2 supplementation. Also, 100 IU/mL IL-2, considered here as the current standard often used in T-cell production [36], generated on average a 2.5-fold smaller proportion of TCM and 5.5-fold more TEff compared with T cells expanded without supplemental IL-2 (P = 0.04 and P = 0.01, respectively; Figure 2).

In conclusion, these results demonstrate that an essential shift toward early memory T phenotypes can be achieved by simply reducing the amount of IL-2 during in vitro T-cell expansion.

Instability of the TSCM population during T-cell expansion

Cell products from altogether 11 donors (healthy n = 9; patients n = 2) were phenotyped for their T-memory status on day 10 of the expansion and, surprisingly, six expansions (55%) were completely deficient in TSCM (Figure 2). Absence of TSCM was not IL-2
**Figure 1.** (A) A representative analysis of T memory phenotyping. T cells were expanded for 10 days under 0 IU/mL IL-2. The T-cell subset composition was 0% naïve, 0% TSCM, 37% TSCM-like, 47% TCM, 15% TEM, and 1% TEff. FMO controls were used for setting the gates. (B) Low IL-2 concentration induces the generation of less-differentiated memory T cells. T cells from peripheral blood were activated and expanded in vitro using CD3/CD28 microbeads with IL-2 concentrations ranging from 0–300 IU/mL. The percentages of early memory T cells, defined as the sum of TSCM, TSCM-like, and TCM cells, were analyzed on the tenth day of the expansion. Cells from five donors were expanded in all the different conditions in parallel. Additional parallel expansions were done for 5–300 IU/mL IL-2 (0 IU/mL n = 5, 5 IU/mL n = 6, 20 IU/mL n = 11, 100 and 300 IU/mL n = 10). Six expansions were lentivirally transduced with CAR and three with GFP. In two expansions, the cells were not genetically modified. Results from individual expansions are shown along with means and 95% confidence intervals. Transparent data points represent patient samples and solid data points healthy donor samples. The Mann-Whitney test was used to calculate statistical significance (*P < 0.05).
dependent, since expansions deficient for T_{SCM} lacked them under all IL-2 conditions (data not shown). Unfortunately, expansions that were also conducted without exogenous IL-2 were, by chance, entirely deficient for T_{SCM}. The proportion of other early T-memory subtypes, i.e., T_{SCM-like} and T_{CM} cells, compensated for the loss of T_{SCM}. Hence, the total proportion of early memory T cells was not significantly lower in cultures where no T_{SCM} were present.

Six expansions, three containing and three deficient for T_{SCM} at day 10, were cultured further and analyzed again at day 20 (Figure 3). The three expansions that did not have detectable T_{SCM} cells on day 10 now had T_{SCM} cells at a mean level of 23% (using 20 IU/mL IL-2). In contrast, the mean T_{SCM} proportion decreased from 43% to 23% by day 20 in those expansions that were already positive at day 10. Contrasting with the instability of the T_{SCM} population, a longer expansion time led to a consistent decrease in T_{SCM-like} and T_{CM} and an increase in the T_{EM} populations (Figure 3). The size of the T_{EM} subset predominantly remained unchanged.

The reason for this variation in the T_{SCM} subset could not be identified but several method-related issues were excluded (e.g., serum batch, culture format [well vs flask], use of fresh vs cryopreserved cells as starting material, laboratory deviations, and type of genetic modification). However, a lower cumulative fold expansion by day 10 was often related to the absence of the T_{SCM} subset at this time point. These findings suggest that the presence of T_{SCM} cells may be linked to expansion kinetics. These data emphasize the sensitivity of primary cell culturing and elucidate the challenges in cell manufacturing.

In summary, the T_{SCM} population was not detected in every expansion product at day 10. However, T-cell cultures that first lacked these cells could bear a prominent population during subsequent expansion.

A shorter in vitro expansion time yields a higher proportion of early memory T cells

Because we found that the proportion of T_{SCM} could change upon prolonged expansion, we next investigated the effect of a longer expansion time on the yield of cells with the desired early memory T-cell phenotype. Using IL-2 concentrations ranging from 0–300 IU/mL, an average 11- to 62-fold expansion of the T-cell product was achieved during the 10-day culture (Table II), and by day 20 of expansion the cells reached 140- to 12 000-fold using 20–300 IU/mL IL-2 (Figure 4). Day 20 data is only available for cultures where 20–300 IU/mL IL-2 was used, because the cells cultured with less cytokine did not proliferate well enough for testing.

Compared with 10 day-long cultures, the day 20 expansion cultures yielded an approximately two-
fold lower mean level of early memory T cells with each tested IL-2 concentration (20–300 IU/mL; Figure 5). Closer subtype analysis in this larger data set confirmed the observations in Figure 3: longer expansion led to a decrease in both T<sub>SCM-like</sub> and T<sub>CM</sub> proportions and increased T<sub>Eff</sub> in all three IL-2 conditions (Supplementary Figure S1). Of note, the T<sub>CM</sub> population was practically lost from expansion cultures by day 20.

These results indicate that, although the total yield of cells is less, shorter in vitro culture may be desirable for clinical T-cell production because it generates T cells with an early memory status.

High IL-2 concentration favors CD4<sup>+</sup> T cells but longer expansion time promotes preponderance of CD8<sup>+</sup> cells

The expanded cell products consisted solely of CD3<sup>+</sup> T cells (>98% on day 10). No γδ T cells were detected, and both CD4<sup>+</sup> and CD8<sup>+</sup> cells were present in all cultures under all conditions. The proportion of CD4<sup>+</sup> cells at day 10 varied considerably between expansions (e.g., proportion of CD4<sup>+</sup> cells ranged from 12%–82% with 20 IU/mL IL-2; Figure 6 and Table II). A high concentration of IL-2 led to a somewhat higher
fraction of CD4. In contrast, longer expansion times favored CD8 over CD4 cells (Figure 6). Under all tested IL-2 conditions (20–300 IU/mL) there was a 2- to 4-fold decrease in CD4 and a reciprocal increase in CD8 proportions from day 10 to day 20. Thus, expansion time had major effect on T-cell populations by altering the cell balance toward CD8 T-cell dominance.

A small fraction of the expanded T cells gained CD56 expression by day 20 of expansion (data not shown). These cytokine induced killer (CIK)-type cells comprised approximately 10% of the cell product, regardless of the IL-2 concentration used (20–300 IU/mL). Similar data have been reported by others but already after 10 days of expansion [36].

In summary, the relative proportions of CD4 and CD8 T cells in in vitro T-cell expansion are affected by both the level of exogenous IL-2 and length of expansion. CD4-enriched products can be generated using a high concentration of IL-2 (≥100 IU/mL) for about 1 week. On the other hand, a longer expansion protocol yields products consisting predominantly of CD8 cells.

**IL-2 concentration affects particularly the memory T-cell subtype distribution of CD4 cells**

We also asked if memory status was differently affected among expanded CD8 and CD4 T cells. In both populations the mean proportion of early memory T cells was high on day 10 under different IL-2 conditions, being 73%–91% for CD8 (Supplementary Figure S2A) and 66%–88% for CD4 cells (Supplementary Figure S2B). Use of high IL-2 concentration during expansion led to a decrease in early memory T cells in both populations as already demonstrated for total T cells (Figure 1B) but the differences reached statistical significance only in the CD4 population. IL-2 induced TEM differentiation in both CD8 and CD4 T cells and lower levels of IL-2 yielded higher TCM numbers at day 10 (Supplementary Figure S3).

If the culture time was extended up to 20 days, only 20%–40% of cells represented early TM subtypes in both CD8 and CD4 cells when 20–300 IU/mL IL-2 was used, and 60%–80% of the cells consisted of TEM and T Eff cells (n = 5, data not shown). At 20 days, increasing IL-2 concentration had a similar effect to what was was seen already on day 10: high concentration of IL-2 (300 IU/mL) favored the generation of higher proportions of TEM (66% and 64%, respectively) within the CD8 and CD4 fractions, whereas less T Eff (50% and 38%, respectively) were generated using 20 IU/mL IL-2. Conversely, high IL-2 concentration led to reduced generation of early TM cells.

In summary, exogenous IL-2 increment in the in vitro T-cell expansion affects particularly CD4 T-cell memory distribution leading to less early memory T cells and a preponderance of differentiated effector cells.

**CD19-CART cells expanded under different IL-2 conditions had versatile in vitro effector functions and were target-specific**

CD19-CAR expression was analyzed at day 10 of T-cell expansion (8 days post-transduction) using flow cytometry (Figure 7A and 7B). The mean percentage of CAR-expressing cells was 52% (range, 8%–89%), and the level of IL-2 supplementation had no apparent effect on transduction efficiency. Both 2G and 3G CAR constructs were successfully expressed on primary human T cells.

Several assays were performed to analyze the impact of varying IL-2 supplementation on the functional characteristics of the CD19-targeted CAR T cells (Figure 7). Buffy coats from three donors (donors 1–3) were used to generate CART cells using the five different IL-2 concentrations (0–300 IU/mL), and the resulting cells were then subjected to proliferation,
IL-2 supplementation during in vitro T-cell expansion

A

% CAR+ cells

IL-2 (IU/mL)

B

Normalized T Mode

CAR AF647

C

% Proliferating cells

IL-2 (IU/mL)

D

% CD107a+ cells

IL-2 (IU/mL)

E

IFN\(\gamma\)

IL-2 (IU/mL)

F

% target cell lysis

E:T ratio

IL-2 supplementation during in vitro T-cell expansion
degranulation and cytokine secretion assays. Transduction efficiency varied between the three donors, being 73% (range, 61%–80%), 51% (range, 21%–66%) and 8% (range, 6%–10%), respectively. In all of the functionality tests, all CD19-CAR T cells showed elevated activity compared with untransduced or empty-LV transduced T cells (Figure 7C–7E), indicating that the observed CAR T-cell functions were dependent on CAR expression. The strength of the anti-B-ALL degranulation, proliferation and cytokine secretion response varied between the three tested CAR T-cell expansions, higher response correlating with the higher proportion of CAR-positive cells (Figure 7C–7E). In all three expansions and regardless of transduction efficiency, the increase of exogenous IL-2 used for CART-cell production led to more active degranulation, proliferation and secretion of IL-2 and IFN-γ in response to target cells (Figure 7C–7E). These results are in agreement with our finding that higher IL-2 concentrations during T-cell expansion yield higher proportions of more differentiated effector T cells.

Cytotoxic potency was measured using the calcein release assay. CD19-CAR T-cell products produced under different IL-2 concentrations all demonstrated efficient and dose-dependent killing of SupB-15 and NALM-6 cells (Figure 7F and Supplementary Figure S4). In addition, CD19-CAR T cells showed minimal cytotoxicity against the CD19-negative B-cell line BC-3 (data not shown), and untransduced and empty-LV transduced T cells showed minimal target cell lysis (Figure 7F and Supplementary Figure S4), confirming that the killing was antigen-specific and dependent on CAR expression. Furthermore, expanding the cells in a high concentration of IL-2 (100 and 300 IU/mL) enhanced the killing of CD19+ target cells compared with cells grown without IL-2 (Figure 7F; P < 0.0001 and P < 0.0004, respectively, by a linear modelling analysis, i.e., analysis of covariance; see supplementary file for details).

In summary, CD19-CAR T cells demonstrated versatile in vitro functions, and the observed T-cell response against B-ALL cells was antigen-specific and dependent on CD19-CAR expression. Moreover, in vitro anti-leukemia response of CD19-CAR T cells was enhanced by the increase of exogenous IL-2 used during T-cell expansion, a change that also favored effector cell differentiation as detected using Tm phenotyping.

Dissection for the clinical CD19-CAR T-cell production protocol

Determining the best expansion conditions for T cells is complex (Table II). Furthermore, also the lowest values in key determinants, not only the average values, are significant for reproducible and cost-effective manufacturing. The appropriate clinical cell dose for CAR T cells is not known and may depend on the cell phenotype in the product. With a target of 2 × 10^6 cells/kg patient weight [4,37], we estimate that a 25-fold cumulative fold expansion in cell number would be needed to cover all patients. On the other hand, only a five-fold T-cell expansion has been used as a highly predictive threshold for successful clinical CART-cell manufacturing [28]. Also, high treatment efficacy with low toxicity was achieved with only 0.2 × 10^6 CD19-CAR T cells/kg with a defined 1:1 CD4:CD8 ratio [38]. Such cell dosing in our estimations corresponds to a three-fold cell expansion only. The total yield for early memory T cells (Table II and Supplementary Figure S5) is pivotal if pure cell populations, and hence also further cell selection, are preferred.

We hypothesize that an effective T-cell product encompasses appropriate subpopulation heterogeneity providing rapid CD8+ effector functions, CD4+ cells and early T memory cells. T-cell expansion without IL-2 supplementation yielded an average of 68% early TCM cells at lowest 83% in 10 days (Table II). The average T-cell subset composition was 0% TSCM, 55% TSCM-like, 32% TCM, 10% TEM and 3% T Eff. In comparison, 100 IU/mL IL-2, the current standard used for T-cell expansion, yielded 15% TSCM, 46% TSCM-like, 13% TCM, 9% TEM and 17% T Eff. Both CD4+ and CD8+ cells were generated, and the cells showed functional potency in vitro. The lowest expansion level without supplemental IL-2 was four-fold but on average 11-fold, corresponding to cell dosing between 0.2 × 10^6/kg–1 × 10^6/kg. By using 100 IU/mL IL-2, the mean cell expansion was 60-fold (range, 3–15; Table II), which would provide cell dosing up to 5 × 10^6/kg. Dose-escalation studies are needed to define the T-cell expansion condition providing the best balance between T-cell subset composition and sufficient total cell number.

Discussion

IL-2, as “the T-cell growth factor” has been used extensively for expansion of T-cells in vitro. Its use has been challenged in recent years as the role of persistent T-cell memory for the clinical success of cancer treatment has gained focus. Thus, the impetus to control effector cell differentiation and memory formation is high. We show here that significant numbers of less-differentiated memory T cells can be generated simply by limiting the IL-2 concentration during cell expansion. Use of higher amounts of IL-2 reduced the generation of early TCM by decreasing central memory T cells and simultaneously increasing differentiated effectors.

To our knowledge, systematic analysis of IL-2 supplementation for in vitro CART T-cell expansion has
not been reported. Here, we have analyzed the association between IL-2 concentration, expansion kinetics and memory T-cell formation using the CD3/CD28 microbead protocol for T-cell activation. Our data demonstrate that conditions that predominantly rely on autonomous IL-2 production (0 and 5 IU/mL) induced T-cell proliferation for only 10 days resembling the expansion kinetics of a normal immune response and by that time yielded the highest proportions of early T memory cells. Low IL-2 concentration (20 IU/mL) still limited cell proliferation to 2 weeks. Higher IL-2 concentrations, that offered T cells seemingly an unlimited source of proliferative signals (100 and 300 IU/mL), led to the accumulation of effector cells already by the tenth day of expansion. One hundred or 300 IU/mL IL-2 is commonly used for in vitro T-cell expansion and even higher supplementation is used for TILs [39,40]. Thus, it may be that in vitro conditions that override the natural physiological course of memory T-cell formation, e.g., high IL-2 concentration, may not be able to generate a significant memory cell pool.

In addition to the present study, two publications have reported in vitro T-cell expansion without any cytokine supplementation. The first one compared two common CART-cell culturing methods used in early trials: soluble anti-CD3 with 300 IU/mL IL-2 to CD3/CD28 microbeads without any exogenous IL-2 [13]. They showed that the bead-based protocol produced a younger T-cell phenotype with greater proliferative capacity and better performance in an in vivo model. Still, it was not clear if the improved results were obtained due to the CD28 cosignal, provision of more physiological activation by bead-bound instead of soluble antibodies or lack of IL-2 supplementation. Our results here indicate that omitting IL-2 was at least partly responsible for the better outcome. The second report investigated the occasional poor in vitro expansion potential of T cells derived from patients with B-cell malignancies [28]; one-fourth of patients were excluded from a CD19 CAR clinical trial because of unsuccessful T-cell expansion. Furthermore, the presence of naïve or early T memory cells (T_{SCM} and T_{CM}) correlated with successful expansion. Together with our results, these studies demonstrate that in vitro T-cell expansion without any cytokine supplementation provides persistence-related early T_{M} cells with increased in vivo anti-tumor efficacy both in the pre-clinical and clinical setting and provides a feasible method for most patients with B-cell malignancies.

T-cell expansion without IL-2 naturally affected the final cell numbers. In our material mainly from healthy donors (two leukemia patients were included), we were able to improve the cell yield without markedly compromising the proportion of less-differentiated memory cells by slightly elevating the IL-2 to 20 IU/mL. This improved the mean cell numbers four-fold by day 10 of the expansion. Higher IL-2 concentrations, namely 100 and 300 IU/mL, further increased the mean cell yield by six-fold compared with expansions relying on autonomous IL-2 production only. Yet, the expansion efficiency is likely lower with patient-derived cell material, although such difference was not observed in this study. Efficient expansion in the absence of IL-2 can also be obtained using IL-7 and IL-15, which was needed especially for patients with lymphoma [28].

IL-7 and IL-15 have been explored in in vitro T-cell expansion with a goal to generate early T_{M}. In normal donors early T_{M} or CD45RO\(^{+}\) T_{SCM} (i.e., T_{SCM-like} cells) alone were increased 10%–30% compared with expansions relying on a high IL-2 concentration (100–300 IU/mL) [25,27,30]. Here we accomplished an increase of similar size (5%–30%) in early T_{M} simply by limiting IL-2 supplementation from the current standard. Compared with no cytokine supplementation, IL-7 and IL-15 induced a 10%–20% gain in the number of T_{SCM} in products derived from cancer patients’ cells. Singh et al. also revealed a simultaneous IL-7 and IL-15–induced decrease in T_{CM} and, interestingly, an increase in T_{Eff} [28]. T_{SCM-enriched} CAR T-cell products can also be produced by combining careful cell selection technology with use of homeostatic cytokines and signaling inhibitors [32]. To conclude, our method using a limited amount of IL-2 during T-cell expansion offers a simple, less manipulative and cost-effective alternative for increasing the proportion of less-differentiated memory T cells.

Among other early T_{M} subsets, we were able to produce T memory stem cells (CD95\(^{-}\)CD45RO CD45RA\(^{-}\)CD27\(^{+}\)). T_{SCM} generation was not IL-2 dependent. Although T_{SCM} were not present in every expansion at a specific time point, each culture that was analyzed contained these cells either on day 10 or day 20. Furthermore, cultures lacking T_{SCM} on the tenth day of expansion proliferated poorly during the first week of expansion, possibly linking the presence of T_{SCM} to expansion kinetics. It is somewhat perplexing how a culture lacking naïve cells and T_{SCM}, the two least-differentiated T-cell subsets, can contain T_{SCM} later during expansion. However, it is possible that these subsets were present but below our detection limit. Also, it is not firmly established if memory T-cell generation follows the progressive differentiation model where less-differentiated subsets function as precursors for more differentiated ones [41].

Most in vitro expansion protocols lack the ability to generate T_{SCM} but produce so-called T_{SCM-like} cells that have gained the expression of CD45RO [25–27,30]. The T_{SCM-like} population was likewise the major memory subset present using our expansion protocol. It is not currently known which of the early T
memory subsets \((T_{SCM}, T_{SCM-like} \text{ or } T_{CM})\) provides the best clinical efficacy in humans. A certain level of cell subset heterogeneity can be a good strategy. Thus, lengthening the expansion time to increase the \(T_{SCM}\) proportion is probably not feasible as the proportion of total early memory T cells decreases. However, finding out the factors inducing the generation of \(T_{SCM}\) in our system would be of substantial scientific interest. Such factors may also have potential in the production of adoptive T-cell products. T-cell–derived natural components may facilitate development of less manipulative ways to produce \(T_{SCM}\) cells. IL-7 may provide key signals for the formation of \(T_{SCM}\)-like cells together with IL-15 [25] and of \(T_{SCM}\) cells together with IL-21 and additional modification of intracellular signaling [32]. IL-7 or IL-15 is not produced by T cells themselves, but IL-21 and IL-9 are produced by activated CD4\(^+\) T cells [24]. IL-9 supports T-cell growth during late phases of immune responses. Interestingly, it also supports anti-tumor activity and survival of TIL through apoptosis resistance [42]. We do not know if the cells in our expansion protocol produced IL-9 or IL-21. However, knowing that early T\(_M\) and CD4\(^+\) cells correlate with T-cell persistence in a clinical setting [10] and that CD4\(^+\) cells are a prerequisite for mouse memory T-cell formation [19,20], it is tempting to hypothesize that CD4\(^+\) cells might play a role in \(T_{SCM}\) generation. As growth kinetics played a role, we hypothesize that \(T_{SCM}\) generation is induced by factors produced by CD4\(^+\) T cells after their peak proliferation.

The significance of CD4\(^+\) cells was recently demonstrated in a sophisticated CAR T-cell study [14]. The authors postulated that IL-2 produced by CD4\(^+\) cells drove the enhanced CD8\(^+\) proliferation in vivo that correlated with efficacy. According to their data, 60% of CD8\(^+\) and 75% of CD4\(^+\) cells expressed CD45RO and CD27 and thus represented early T\(_M\). A similar cellular distribution was obtained with our simplified method in a 10-day expansion using IL-2 at 20–100 IU/mL. Due to the prominent role of CD4\(^+\) T cells in adoptive T-cell therapy, a better understanding of their optimal proportion and how expansion conditions impact their numbers in relation to CD8\(^+\) T cells is needed. We show that the proportion of CD4\(^+\) cells is increased if very low or high concentrations of supplemental IL-2 are used, but that extending the expansion time from 10 to 20 days leads to the accumulation of CD8\(^+\) cells at the expense of CD4\(^+\).

Several studies indicate that improved in vivo performance in humans [9,10] and animal models [11–14] is achieved with T-cell products that contain early memory T cells, either \(T_{SCM}, T_{SCM-like} \text{ or } T_{CM}\). In addition to memory T-cell phenotyping, we showed that the cell products expanded under different IL-2 conditions had differences in in vitro function. Although the tests do not measure persistence, the results demonstrate that the products differ functionally. For these reasons, even though these studies did not include in vivo testing, it is reasonable to infer that improved in vivo performance may be achieved using the cells produced with low IL-2 supplementation as well.

One more consideration regarding the use of early memory T cells in the treatment of cancer remains unresolved. Assuming that in vitro–generated early memory T cells have a similar homing capacity to their physiological counterparts [12,15], they will have limited potential to enter peripheral tissues such as tumors, and rather home to lymphoid organs for activation by antigen-presenting cells. In cancer, the function of antigen-presenting cells is often suppressed. Thus, early memory cell–enriched T-cell products may be particularly effective in hematologic malignancies where malignant target cells are found in lymphoid organs, but less so in solid tumors lacking lymph node metastasis.

Less-differentiated memory T cells are critical for clinical anti-tumor efficiency. We demonstrate that the number of early T\(_M\) cells in a T-cell preparation can be increased by simply reducing the IL-2 amount used during in vitro T-cell expansion. In light of our results, the necessity of sophisticated multi-step protocols may need re-evaluation. These findings are significant for robust and cost-effective clinical T-cell manufacturing.

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References


Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2017.03.067.