T cell Therapy After Allogeneic Stem Cell Transplantation

Titration of IL-2 Concentration When Expanding Cord Blood T Lymphocytes With and Without IL-7, In Order to Optimize Expansion Protocol for Donor Lymphocyte Infusion

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ABSTRACT

Umbilical cord blood (UCB) is a promising, increasingly used option for allogeneic stem cell transplantation. Among the beneficial features are availability, permissiveness regarding human leukocyte antigen (HLA)-matching, and low incidences of graft-versus-host disease (GVHD). A great disadvantage is the low cell number in the grafts and therefore insufficient T cell numbers to prepare for donor lymphocyte infusion (DLI). Expansion of cord blood cells in vitro has been conducted to solve this obstacle, and in this paper we present an attempt to optimize the expansion protocol used by Okas et al. for expansion of T cells for DLI. By using different culture conditions in five UCB derived T cell expansions, the results showed propitious effects of interleukin (IL)-7 and that an IL-2 concentration of 100-200 IU per ml medium induce the most effective expansion of T cells.

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LIST OF ABBREVIATIONS

aGVHD	Acute graft-versus-host disease
AICD	Activation-induced cell death
ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia
ASCT	Allogeneic stem cell transplantation
BM	Bone marrow
BMSCT	Bone marrow stem cell transplantation
BMT	Bone marrow transplantation
CB	Cord blood
CBU	Cord blood unit
CD	Cluster of differentiation
cGVHD	Chronic graft-versus-host disease
CLL	Chronic lymphoid leukaemia
CML	Chronic myeloid leukaemia
DLI	Donor lymphocyte infusion
EFS	Event-free survival
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FasR	Fas receptor
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukaemia
HD	Hodgkin's disease
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
MDS	Myelodysplastic syndrome
MM	Multiple myeloma
MNC	Mononuclear cell
NC	Nucleated cells
NHL	Non-Hodgkin's lymphoma
NMC	Non-myeloablative conditioning
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RCC	Renal cell carcinoma
RIC	Reduced intensity conditioning
SC	Stem cell
SCF	Stem cell factor
SCT	Stem cell transplantation
T cell	Thymus-dependant lymphocyte
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
T_H1	T helper type 1
T_H2	T helper type 2
TKI	Tyrosine kinase inhibitor
Tn	Naïve T cell
TNC	Total nucleated cells
T_{reg}	Regulatory T cell
TRM	Transplant related mortality
Ttde	Terminally differentiated T cell
UCB	Umbilical cord blood
UCBT	Umbilical cord blood transplantation

1. INTRODUCTION

1.1. Indications and Stem Cell Sources for Allogeneic Stem Cell Transplantations

Allogeneic stem cell transplantations (ASCTs) have been used as therapy since 1957 (Thomas et al.), although reports of use of bone marrow infusions for treatment of anaemia and leukaemia extend to late 19th century. It is today a well-established therapy for haematological and oncological diseases. Current indications for ASCT include acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS), and some acute lymphoid leukaemias (ALLs) in patients with poor response to chemotherapy alone. Another, former indication was chronic myeloid leukaemia (CML) until the introduction of tyrosine kinase inhibitors (TKI). Now ASCT is administrated only after treatment failure of TKIs. ASCT is also used to treat chronic lymphocytic leukaemia (CLL), multiple myeloma (MM), myelofibrosis, high-risk lymphomas, Hodgkin's disease (HD), non-Hodgkin's lymphoma (NHL). As a more experimental approach ASCT have been tried as a treatment modality for some metastatic solid tumours, including renal cell carcinoma (RCC), prostatic cancer, carcinoma of the ovaries, breast and pancreas, although with variable results. The non-malignant indications include severe aplastic anaemia, some haemoglobinopathies and some inborn errors of metabolism and immunodeficiency syndromes [1-6].

The indications for ASCT are constantly evaluated and changed. The introduction of non-myeloablative conditioning (NMC) or reduced intensity conditioning (RIC) has included older patients and patients with co-morbidities that traditionally would be selected out. [7-10]

Traditionally, the source of stem cells was bone marrow (BM). After the later implementation of retrieving haematopoietic stem cells from peripheral blood mononuclear cells (PBMC), the term haematopoietic stem cell transplantation (HSCT) was established [11]. Today, PBMCs is the first choice as stem cell (SC) source in adults, since no anaesthesia is needed and because of the easy availability of more haematopoietic stem cells (HSCs). This is also supported by results showing that engraftment, frequency of graft failure, relapse, acute graft-versus-host disease (aGVHD) and survival are comparable to BM, although frequency of chronic GVHD (cGVHD) is increased [12-15].

For both malignant and non-malignant conditions, the approach to ASCT starts with human leukocyte antigen (HLA)-typing of siblings. HLA typing is today performed in a PCR-based manner, with DNA sequence-specific typing techniques. The genotype results are presented in one of two ways; low-resolution- and high-resolution. Low resolution (2-digit typing) corresponds to the serological typing used before, and identifies broad families of alleles, while high-resolution typing (4-digit) identifies the individual alleles within the serotype [16, 17]. HLA-disparity can have detrimental effects on the

outcome of SCT and leads to alloimmune responses in either direction, i.e. graft-versus-host or host-versus-graft, that can lead to GVHD or graft failure, respectively [18].

If no HLA-identical sibling is found, the search continues in national and international registries. In children with leukaemia who have HLA-identical sibling donors, BM is the first choice as source of stem cells, after a retrospective study showed increased occurrence of cGVHD and mortality with the use of PBMCs [19]. In about 1/3 of cases, no suitable donor can be found in the stem cell registries around the world [20].

If no identical unrelated donor can be found, i.e. a 10/10 match (HLA-A, -B, -C, -DRB1, -DQB1 – gold standard) or a 6/6 match (HLA-A, -B, -DRB1 - minimum matching level accepted), a mismatched donor has to be utilized. Umbilical cord blood (UCB) is a source of HSCs with less stringent demands of HLA matching. UCB is increasingly used as a source of stem cells: in 1993 the number of UCB transplants (UCBT) was 3 while the number in 2008 was 3 529. The availability of cord blood units (CBUs) has increased 10-fold the last 10 years: from 44 000 banked CBUs in 1999 to 452 000 in 2008 [21]. The current number of banked units is > 470 000 (http://bmdw.org/).

A 4/6 HLA match has been shown to have comparable results to matched and mismatched (1-2 HLA) unrelated BMSCT and also matched sibling donors [22-24]. HLA matching for UCB is defined from low resolution for HLA-A and -B and high resolution for HLA-DR.

It has been, and is still discussed whether HLA-match or cell dose has the greatest impact regarding the outcome of UCBT. It has been shown that low cell dose, which often is a problem with UCB, is associated with delayed engraftment and increased transplant related mortality (TRM) [25]. Also with higher degree of HLA mismatch, the cell dose is of greater importance [26].

One way to overcome the cell dose problem, which has been employed lately, is the transplantation of two UCB-units, partially matched to the recipient and between each other [5, 27, 28].

The permissiveness of HLA matching of CB units is a feature that has been shown in multiple studies, and also that potential mismatch(es) can partly be compensated for with increased cell dose, and that cell dose is the most important factor for outcome [29-31]. Other studies argue for HLA matching as the main predictor of outcome [32, 33]. Several studies have, however, demonstrated and concluded that cell dose is more important than HLA-matching in order to achieve a successful transplantation [25, 34-37].

The study by Gluckman et al. of EUROCORD data concluded that there was a decreased negative effect of HLA mismatches when compensated for with a higher cell dose. These findings were evident when using grafts with 1-2 HLA mismatches. Patients receiving grafts of 3-4 HLA-mismatches were not influenced by increased cell dose [31]. They concluded with the following recommendations of donor selection and number of nucleated cells (NC) in the graft:

1. 6/6 match with cell dose > 3 *10^7 NC/kg
2. 5/6 match with cell dose > 4 *10^7 NC/kg
3. 4/6 match with cell dose > 5 *10^7 NC/kg

The outcomes of UCBT compared to BMT are promising. Multiple studies have shown that engraftment after UCBT is delayed, with a longer period of neutropenia and associated increased risk of bacterial infections. The prolonged lymphopenia seen after UCBT and the naivety of CB T cells also contributes to the increased rate of opportunistic infections seen after UCBT, which is the major cause of TRM in the first 6 months post-transplant [38]. However, the current clinical results of UCBT compared to BMT, shows comparable results regarding overall survival (OS), event-free survival (EFS), incidence of relapse and a similar or decreased risk of GVHD [22-24, 39-43].

As mentioned, cord blood grafts have shown to induce GVHD to a less degree, both in incidence and severity. This is probably due to a more naïve T cell population, that have been shown to have a smaller capacity to produce cytokines [44] and perform lysis, shown by measuring cytotoxic activity in mixed leukocyte cultures [45].

Multiple definitions of T cell subsets have been proposed based on the expression of certain molecules. We use the definition that follows: Naïve T cells are cells that have left the thymus and not yet met their antigen. They are termed Tn, express CCR7 (a lymph node homing receptor) and do not express CD45RO. T cells positive for both, i.e. $CCR7^+$ $CD45RO^+$ are termed central memory T cells (Tcm), when they lose their constitutive expression of CCR7 and still are $CD45RO^+$ they are effector memory T cells (Tem), while T cells negative for both are terminally differentiated effector T cells (Ttde) (Table 1). Tcm compared to Tn cells are less dependent on co-stimulation and are more sensitive to antigenic stimuli, while Tcm compared to Tem have little effector function and can easily proliferate and differentiate into effectors when stimulated by antigens in secondary lymphoid organs and Tem moves out to the inflamed tissues to display their effector functions [46, 47].

	T Cell Subsets			
	Naïve	Central Memory	Effector Memory	Terminally Differentiated Effectors
CCR7	+	+	-	-
CD45RO	-	+	+	-

Table 1: Surface molecule expression of the different T cell subsets.

1.2. Umbilical Cord Blood as Source for ASCT

The latest established alternative for ASCT is umbilical cord blood (UCB). The first successful cord blood transplantation was executed in 1988 by Gluckman et al. in a patient with Fanconi's anaemia who received umbilical cord blood from his sister [48].

UCB is today most often used when no HLA matched donor is found, since 1-2 HLA mismatches can be tolerated with the use of cord blood. The matching of UCB is currently defined with low resolution assays for HLA-A and HLA-B, and high resolution assays for HLA-DR. Because of the permissiveness for mismatch, the probability of finding a suitable donor is higher, also for patients with uncommon tissue types. In 99% a 4/6 match will be found and in 70% a 5/6 or 6/6 match [49]. Other features that favours UCB as a stem cell source is its availability; the product is frozen and ready to be thawed and used, it has been HLA typed, serological status for multiple viruses and other infectious agents is known and the number of total nucleated cells (TNCs) has been established.

1.3. T Lymphocytes

Donor lymphocyte infusion (DLI) has been used as adoptive immunotherapy for threatening malignancy relapse, graft failure, and post-transplant infections after SCT [50-53]. T cells are responsible for the majority of the graft-versus-leukaemia (GVL)-effect seen after ASCT. This has been supported by the observed effect of administration of DLI after transplantation, where eradication of relapsing malignancy can be achieved [52, 54-56]. DLI can also induce GVHD in as much as 50 % of patients receiving DLI. It is still not possible to segregate GVHD from the GVL effect. For UCB-transplants, DLI has not been a possibility for logistical and ethical reasons. To overcome this obstacle, expansion of T cells in vitro from the cord blood graft has been conducted. At the time of cord blood sampling, approximately 5% of the graft has been taken out for the possibility of expansion of T cells in vitro. GVL effect, its association with GVHD, and T cells as the main effectors of the response, have been advocated for through results that showed lower relapse rates in patients who developed GVHD [57]. Also, the higher risk of relapse seen in patients receiving syngeneic transplants compared to matched sibling donors [58, 59] and the increased relapse rates in patients receiving T cell depleted grafts [60] has supported this.

Ex vivo expansions of T lymphocytes have been described with procedures using anti-CD3/-CD28 beads and interleukin (IL)-2, originally developed for expansions of T lymphocytes from PBMC [61-63]. Other protocols, including the use of IL-7, anti-CD3, IFN-ɣ, IL-12, IL-4, Stem cell factor (SCF) have been published [64-67].

1.4. IL-2 and IL-7: Two Members of the γ_c Family Cytokines

The γ_c family cytokines consists of the interleukins IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. They share the γ_c component of their receptors, also known as IL-2Rγ and CD132 [68-74]. The sharing of receptor components is a part of a complicated system of overlapping effects and cross-regulation between these cytokines (reviewed in [75]).

Most of these cytokines are produced by cells of the immune system after different stimuli, while IL-7 and IL-15 are constitutively produced by stromal and epithelial cells in bone marrow and thymus and by fibroblastic reticular cells in the T cell zones of secondary lymphoid organs [76, 77].
IL-7 is of uttermost importance in the regulation of naïve and memory T cell homeostasis [78-81]. Since IL-7 is constitutively produced, its effects are regulated through the expression of IL-7Rα, that is expressed by naïve and memory cells, and down regulated after activation through TCR, and by IL-7 itself, IL-2, and other cytokines with pro-survival properties [78, 82-84]. This supports the homeostatic properties of IL-7 in that it leaves more of the continuous IL-7-pool for cells that have not received a signal for survival and increases the availability of the cytokine for these [84]. Direct effects of IL-7 signalling on T cells are promotion of survival through pro-survival PI3-kinase-AKT signalling pathway and increased expression of several survival factors and inhibition of pro-apoptotic factors expression[76, 85]. In addition IL-7 also induces proliferation of memory T cells in physiological conditions and memory- and naïve T cells under lymphopenic conditions. [78, 79, 86, 87]

IL-2 is a cytokine with many functions. The IL-2 receptor (IL-2R) has three subunits, IL-2Rα, IL-2Rβ and the γ_c. They form the high-affinity IL-2 receptor. Low-affinity and intermediate-affinity versions exist in other combinations of the subunits [88, 89]. IL-2 induce T cell proliferation and differentiation (reviewed in [90]) and is involved in the formation of regulatory T cells (T_{reg}). Although IL-2 is one of the main products of T_H1 cells [91], it is required for induction of differentiation into T_H2 cells through an increased responsiveness to IL-4 by promoting expression of the IL-4Rα [92]. Summarized, IL-2 is immunostimulatory to T cells in the following ways: support of T cell proliferation [93-98], support of T cell survival [99] and it drives T cell differentiation from naïve T cells to effector and memory cells [100-102].

1.5. Aims

We wanted to improve the method used for T cell expansion of UCB T cells by Okas et al. [103] by varying the concentration of IL-2 and adding IL-7 in 8-day cultures.

2. MATERIAL AND METHODS

2.1. Antibodies and Reagents

Fluorescein isothiocyanate (FITC)-labeled anti-CD3, phycoerythrin (PE)-stained Annexin V, 7-AAD (both from BD Pharmingen apoptosis detection kit), PE-anti-CD3, PE-Cy7-anti-CCR7, PE-Cy5-anti-CD95, PE-Cy5-anti-CD3, PE-Texas Red-anti-CD4, allophycocyanin (APC)-labeled anti-CD45RO, APC-anti-CD4, APC-Cy7-anti-CD8 (BD, Pharmingen, USA).

CD3/CD28 beads (Dynabeads ClinExVivo, Invitrogen Dynal AS, Oslo, Norway), CliniMACS phosphate buffered saline (PBS)/ethylene diamine tetra-acetaten (EDTA) buffer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), Lymphoprep (Fresenius Kabi Norge AS), DMSO and RPMI 1640 (SigmaAldrich, Germany).

2.2. Expansion of Cord Blood-Derived T Cells

Five cord blood units were received from the maternity ward on Karolinska University Hospital Huddinge, Stockholm.

The method described by Okas et al. for clinical expansion has been used as a basis [103].

Before freezing, the cord blood was diluted 1:1 with PBS and the CB mononuclear cells (MNC) were separated using density gradient centrifugation in Lymphoprep. MNC were pipetted off and washed 3x in PBS. After counting, the cells were frozen in RPMI 1640-medium with 10 % DMSO, to inhibit forming of ice crystals.

The day of thawing and washing of cells, termed day 0 of the expansion, a 5 % aliquot of the CB unit were thawed and washed once in RPMI 1640 medium and thereafter once in PBS/EDTA buffer. Further the cells were counted and re-suspended in PBS buffer with the same amount of anti-CD3/-CD28 magnetic beads (after they were washed x1 in PBS to remove detached antibodies) as the amount of MNC, which varied in our five expansions from $64*10^6$ to $94*10^6$ cells. The cells and beads were incubated for 30 minutes in refrigerator at 4 °C, the T lymphocytes could then be positively selected on a magnet. The positively selected cells were then placed in incubation medium, consisting of RPMI 1640-medium, complemented with 2mM L-glutamine (Sigma Aldrich Inc., St. Louis, Missouri) 100 IU/ml Penicillin G (antibiotic), 100 µg/ml Streptomycin (antibiotic), 0.25 µg/ml Amphotericin B (antifungal drug) (Invitrogen Corp., Carlsbad, California) and 10% pooled human serum (Department of Transfusion Medicine at Karolinska University Hospital, Huddinge). The different culture conditions and their concentrations of IL-2 and IL-7 are shown in table 2. Only the first of our five expansions had all fourteen conditions, the last four were expanded in conditions numbered 1-12, i.e. up to an IL-2 concentration of 600 IU/ml.

Culture Condition no.	IL-2 conc. [IU/ml]	IL-7 conc. [ng/ml]
1	0	0
2	0	20
3	50	0
4	50	20
5	100	0
6	100	20
7	200	0
8	200	20
9	400	0
10	400	20
11	600	0
12	600	20
13	800	0
14	800	20

Table 2: Each culture condition with their respective concentrations of IL-2 and IL-7 that were used in the expansions.

The cells were incubated at 37 °C with 5% CO_2 and kept at concentrations ≤ 300.000 cells/ml throughout the expansion period. From day 0 through day 3 the cells did not proliferate enough to be diluted, though on day 4, 5 and 6 the cells were counted. Medium, IL-2 and IL-7 were refilled to keep stable concentrations. Day 7, the day of finalization, the cells were once again counted, washed twice on magnet (the cells and beads are at this point of time separated) and frozen in 10 % DMSO freezing medium at concentrations < 10.000.000 cells/ml.

2.3. Cell Surface and Intracellular Staining for Flow Cytometry

The day of fluorescence-activated cell sorting (FACS) analysis the cells were thawed and washed as described earlier. The cells were then equally distributed into wells of a 96-well cell culture plate where the antibodies for cell surface markers were added with staining buffer and PBS and incubated in refrigerator at 4 °C for 30 minutes before washing x2 and re-suspension in PBS.
Annexin V/7-AAD staining was executed following the instructions from the manufacturer (BD Pharmingen). FACS was performed using the LSR II Flow Cytometer System (BD Biosciences, USA), and the data were analysed using the FlowJo software (Tree Star, Inc., Oregon, USA).

2.4. Statistical Analysis

Data was analysed and displayed using the Excel software (Microsoft Corp., Redmond, Washington, USA).

3. RESULTS

3.1. Fold Expansion by T Cells

The protocol described was used in five expansions, with good and quite unambiguous results. The numbers of T cells in our expansions were at day 0, the first day of incubation, between $2.05*10^6$ and $3.85*10^6$ cells. The T cells were incubated for 8 days (day 0-7), and expanded with an average fold expansion of 65.1 in all the conditions, and by 50.0 and 80.3 for the culture conditions with only IL-2 and IL-2 plus IL-7, respectively. When adding IL-7, the expansion was highest in culture condition 6 and 8, with 100 and 200 IU/ml IL-2, respectively. IL-7 induced in average 62% increased fold expansion. Quite clear tendencies were observed, although no statistical significance could be demonstrated.

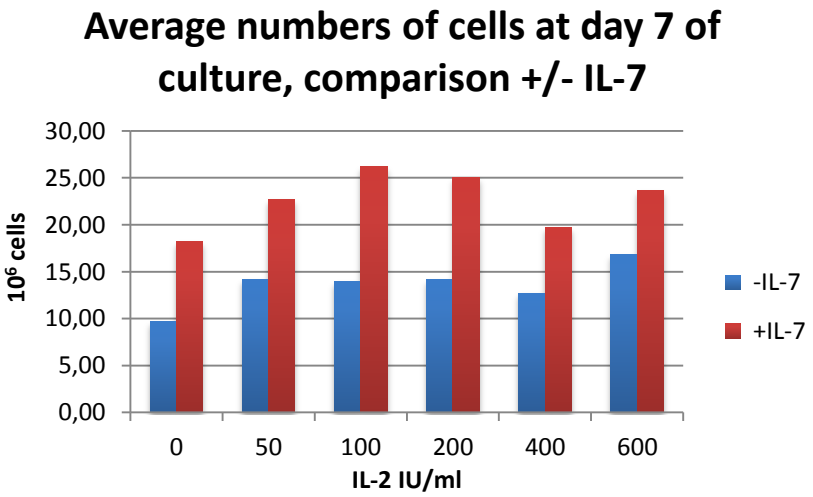

Figure 1a: Average number of cells after an 8 day culture, comparison of each IL-2 concentration +/- IL-7.

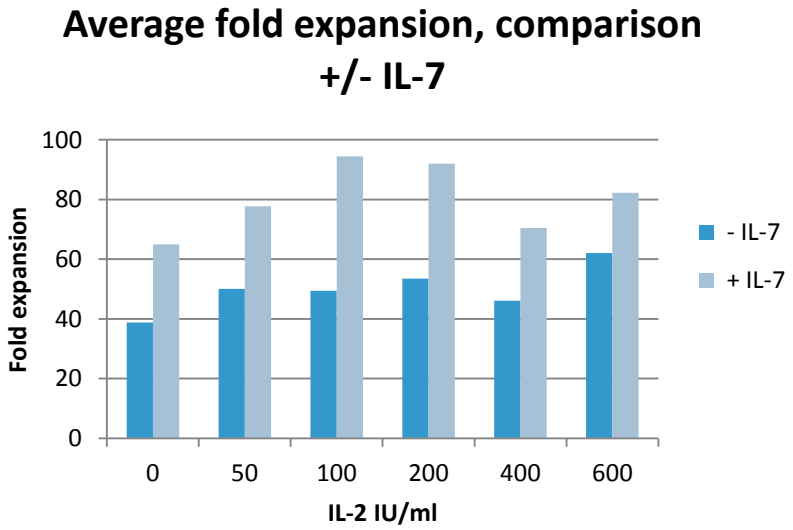

Figure 1b: Average fold expansion after an 8 day culture, comparison of each IL-2 concentration +/- IL-7.

3.2. CD4+/CD8+-Ratio of Expanded T Cells

The expanded cells had an average $CD4^+/CD8^+$-ratio of 2.56, with average ratios of 2.18 and 2.95 for culture conditions without and with IL-7, respectively. Although no significant differences, the average ratios for each culture condition are given in figure 2.

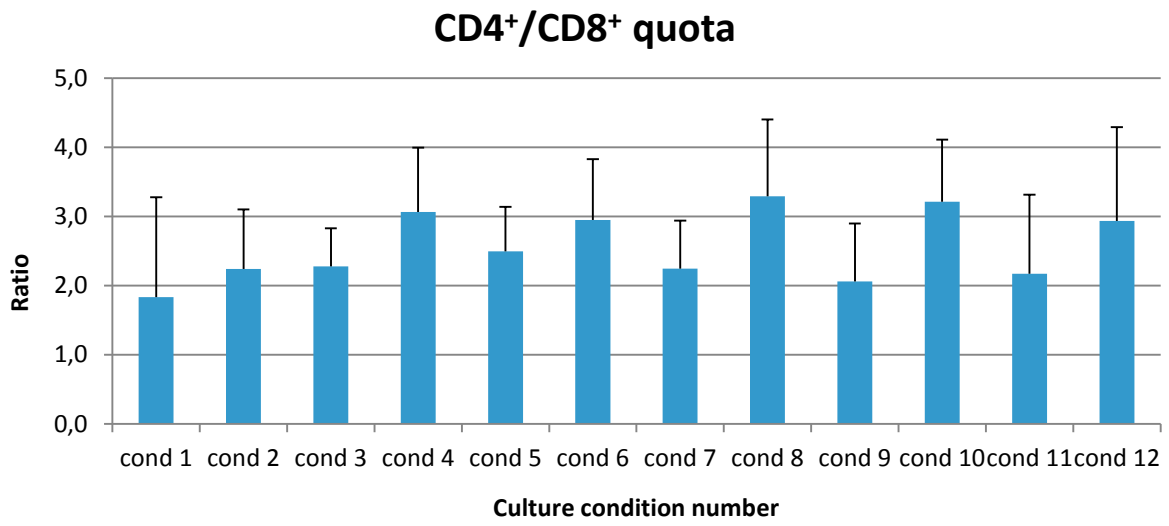

Figure 2: Average $CD4^+/CD8^+$-ratios for each culture condition of the expansions, standard deviations are shown in error bars.

3.3. Degree of Differentiation in Expanded T Cells

Based on the definitions of naïve cells, effector cells and memory cells above, the CD3+ cells could be grouped according to expression of CCR7 and/or CD45RO. Our data showed that most of the expanded $CD3^+$ cells are of the central- and effector memory cell subsets (Tcm and Tem). Also a considerably amount of the expanded cells in culture condition 1, i.e. no IL-2 and no IL-7, were of the naïve subset (22.3%). It was an evident difference in memory subsets in culture conditions receiving IL-7 from those that did not. Most clearly seen in $CD4^+$-cells, Tem cells were a bigger portion of the expanded cells in cultures with IL-7, and in cultures that only received IL-2, the proportion of cells of Tem and Tcm subsets were similar. These differences were not as evident among the $CD8^+$ cells, but the differences can be seen in the overall $CD3^+$ pool. The $CD8^+$ portion of cells did however have larger quotas of naïve (Tn, $CD45RO^-CCR7^+$) and terminally differentiated (Ttde, $CD45RO^-CCR7^-$) cells than the $CD4^+$ cells (Fig. 3).

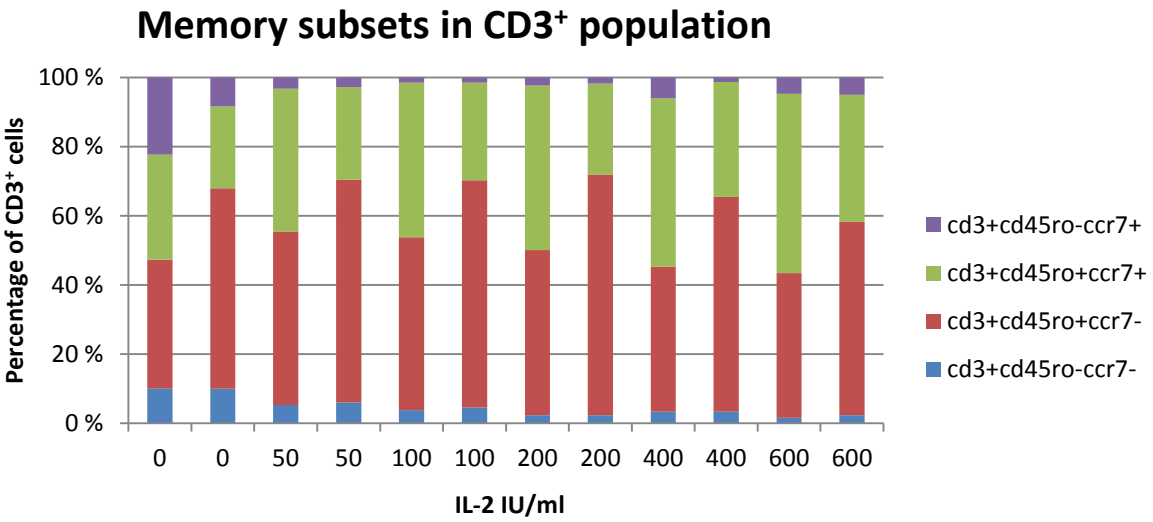

Figure 3a: Distribution of expanded $CD3^+$ cells in subsets of differentiation. Naïve T cells (Tn) $CD45RO^-CCR7^+$. Central memory T cells (Tcm) $CD45RO^+CCR7^+$. Effector memory T cells (Tem) $CD45RO^+CCR7^-$. Terminally differentiated effector T cells (Ttde) $CD45RO^-CCR7^-$.
Conditions 1-12 with their respective IL-2 concentrations are shown the *x*-axis.

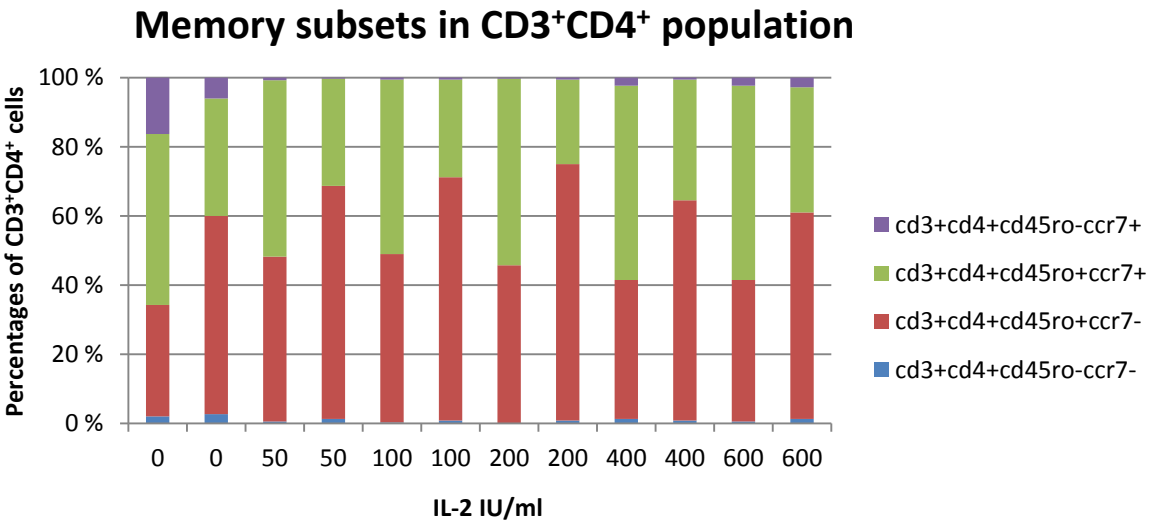

Figure 3b: Distribution of expanded $CD3^+CD4^+$ cells in subsets of differentiation. Naïve T cells (Tn) $CD45RO^-CCR7^+$. Central memory T cells (Tcm) $CD45RO^+CCR7^+$. Effector memory T cells (Tem) $CD45RO^+CCR7^-$. Terminally differentiated effector T cells (Ttde) $CD45RO^-CCR7^-$.
Conditions 1-12 with their respective IL-2 concentrations are shown the *x*-axis.

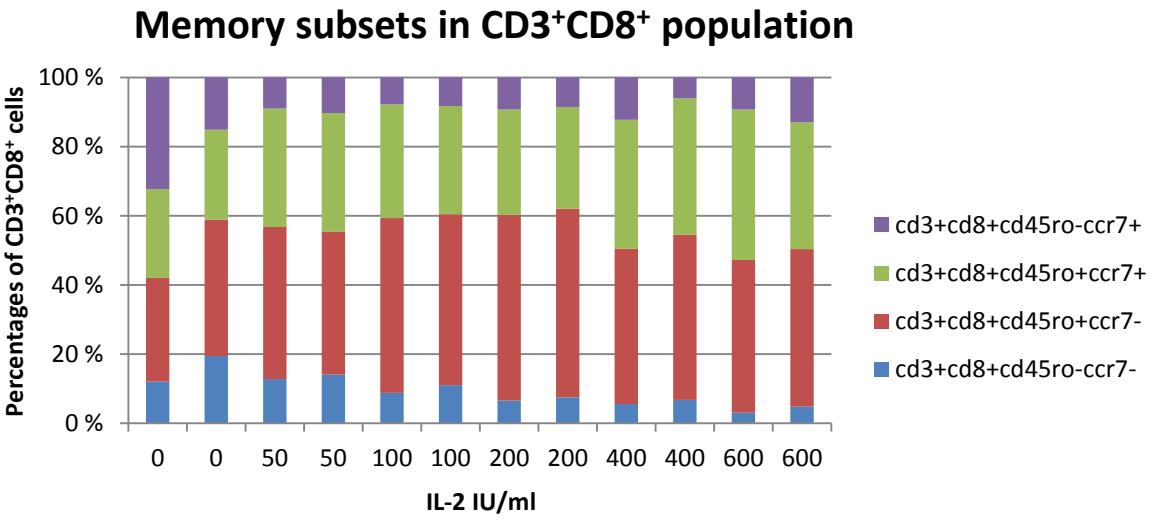

Figure 3c: Distribution of expanded $CD3^+CD8^+$ cells in subsets of differentiation. Naïve T cells (Tn) $CD45RO^-CCR7^+$. Central memory T cells (Tcm) $CD45RO^+CCR7^+$. Effector memory T cells (Tem) $CD45RO^+CCR7^-$. Terminally differentiated effector T cells (Ttde) $CD45RO^-CCR7^-$.
Conditions 1-12 with their respective IL-2 concentrations are shown the *x*-axis.

3.4. Expression of CD95 and Binding of 7-AAD and Annexin V by Expanded T Cells

T cells expressing CD95 (FasR) can easily, by binding FasL, get a signal of apoptosis. CD95/FasR can be up-regulated by T cell activation, IL-2 and anti-CD3 [104-106], and is involved in activation-induced cell death (AICD) [107]. As could be expected, almost all of the expansions had 100% $CD95^+$ cells. The lowest percentage of $CD95^+$ cells was seen in culture condition 1 of the first of our five expansions where 97% of cells were $CD95^+$.

Annexin V is a protein that binds to phosphatidylserine, which is translocated from the inside of cell membranes to the outside upon initiation of apoptosis. We have therefore used this as a marker of early apoptosis. 7-AAD (Amino-Actinomycin D) binds strongly to DNA and cannot pass through intact cell membranes. It can therefore be used to identify cells that are dead; either by necrosis or late stage apoptosis where cell membrane integrity is lost.

The expanded T cells were divided into live cells (Annexin V^- $7\text{-}AAD^-$), apoptotic cells (Annexin V^+) and dead cells, either by apoptosis or necrosis (Annexin V^+ $7\text{-}AAD^+$). In most of the culture conditions there was a tendency to be more alive/viable cells in those receiving IL-7, and in all of the culture conditions there were in average most dead cells in those expanded with only IL-2. The only two culture conditions where live/viable cells were in average a majority, were culture conditions 6 and 8, both with IL-7 and 100 and 200 IU/ml IL-2 respectively (Fig. 4). These were also the same two culture conditions with the highest overall fold expansion (Fig. 1b).

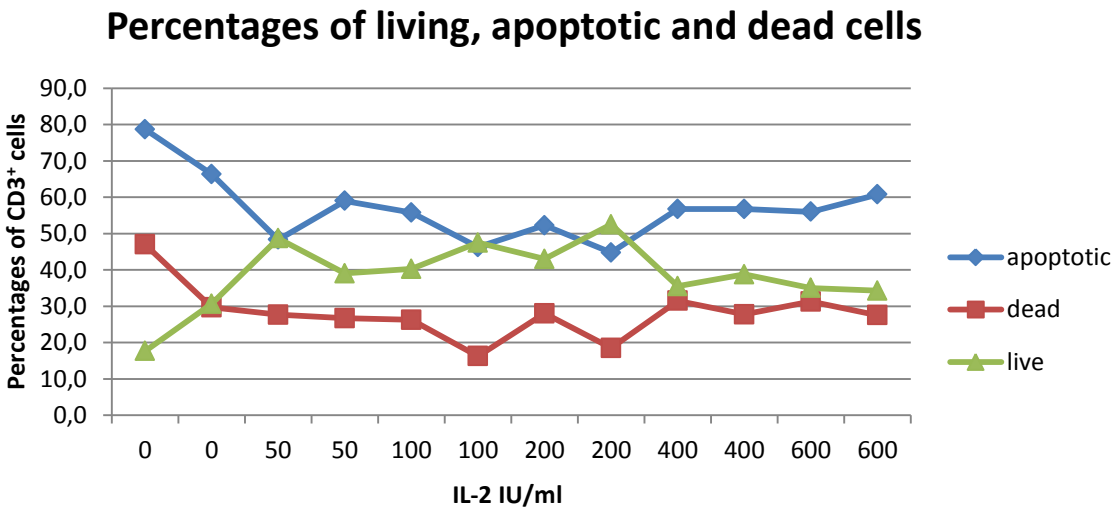

Figure 4: Percentages of expanded T cells that were alive, apoptotic and dead, classified from their binding of Annexin V and/or 7-AAD: Annexin V^- $7\text{-}AAD^-$, Annexin V^+, Annexin V^+ $7\text{-}AAD^+$, respectively.
Conditions 1-12 with their respective IL-2 concentrations are shown the *x*-axis.

4. DISCUSSION

We have only completed five expansions with this protocol, i.e. the 12 different culture conditions described. Due to this low number of expansions, it is difficult to prove any statistical significance of the data. Nevertheless, it seemed to be clear and uniform tendencies of our results.

4.1. Fold Expansion

The data of our expansions shows an advantageous effect of IL-7 on fold expansion. This coincides with data from a pilot project we executed earlier (data not shown) and also with data presented by Davis et al. in 2010 (ref. [108]), although there were some differences between the protocols used: they used an expansion period of 12-14 days, while ours were of 8 days, they operated with an IL-2 concentration of 100 units/ml +/- 10 ng/ml IL-7, and kept a cell concentration of $1*10^6$ cells/ml throughout the expansion period. Davis et al. demonstrated a significant difference in fold expansion.

4.2. $CD4^+/CD8^+$-Ratio

From our data we could suspect a higher $CD4^+/CD8^+$-ratio among cells expanded with IL-7 compared with IL-2 alone. This concurs with our pilot expansions that were performed some time ago (data not shown). Okas et al. (ref. [103]) demonstrated a skewing of $CD4^+/CD8^+$-ratio among in vitro expanded T cells compared to peripheral blood and non-expanded T cells. Mazur et al. showed in 2008 (ref. [64]) an inversion of $CD4^+/CD8^+$-ratio in expansions with IL-2 and CD3/CD28 co-stimulation. This suggests that expansions including IL-7 could decrease/inhibit this skewing or inversion of this ratio.

4.3. Degree of Differentiation

In our experiments there were mainly T cells of memory subsets (Tcm and Tem) among the expanded cells. This correlates well with the data from Okas et al. (ref. [103]) where naïve (Tn) and terminally differentiated effector (Ttde) T cells were not present at all after expansion. The absence of naïve cells could either be a result of lacking proliferation of these, or a differentiation of proliferating naïve cells into memory subsets. These cell subsets were not absolutely absent in our expansions however, especially among $CD8^+$ T cells. In the study published by Davis et al. (ref. [108]) they grouped T cells in differentiation subsets based on other cell markers: “phenotypically naïve” cells were defined as $CD45RA^+CD62L^+$ and, in contrast to our expansions, they showed that these markers were expressed by most expanded T cells, with significant more among the IL-7 cultured T cells (73% versus 90%). Also, they reported that CCR7 was expressed by most of the expanded cells. These

differences in classification molecules make it difficult to compare subsets, since Davis et al. reported that the degree of expression of these molecules were nearly identical to non-expanded cord blood T cells, while data from Okas et al. showed that in non-expanded T cells less than half of the T cells were of a naïve phenotype.

4.4. CD95 Expression and Quotas of Apoptotic and Dead Cells among Expanded Cells

Almost all of the T cells expanded with our protocol expressed CD95/FasR. This could be expected from our culture conditions containing IL-2. This high expression can, in part, explain the high number of apoptotic and dead cells among our expanded T cells, through its involvement in AICD.

Identification of apoptotic and dead cells were based on binding of Annexin V and 7-AAD as described. We could see a tendency towards beneficial effects from IL-7 on expanded T cell survival, as could Davis et al. [108] with demonstration of significantly more viable cells in cultures including IL-7 (71% versus 46%). They had in addition significantly higher numbers of apoptotic cells in absence of IL-7, in both $CD4^+$ and $CD8^+$ subsets. The latter results were based on expression of intracellular activated caspase-3. They also analyzed cells after freezing, thawing and resting in cytokine-free medium, where 53% and 57% of IL-2-only cells stained for 7-AAD and Annexin V, respectively, while the same numbers for cells receiving IL-7 were 33% and 39%.

5. CONCLUSIONS

From our results of five UCB T cell-expansions, it is difficult to promote changes in culture conditions, although our results were mostly uniform in showing the advantageous results of IL-7. The formerly used concentrations of 100-200 IU/ml IL-2 seem adequate for expanding T cells in vitro.

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