T CELL EXPANSION FROM UMBILICAL CORD BLOOD WITHOUT THYMIC STROMA CELLS AFTER STIMULATION WITH SCF, IL-7, AND IL-2

Johannes Fischer[1], Fatemeh Sabagh[2,*], Gesine Kogler[1], Anna R. Migliaccio[3], Peter Wernet[1]

[1]Bone Marrow Donor Center with Eurocord Bank and Transplantation Immunology, Heinrich Heine University, Dusseldorf, Germany
[2]Department of Basic Sciences, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Laboratorio di Biologia Cellulare, Instituto Superiore di sanita, Rome, Italy

Corresponding Author: email address: fatemehsabagh@yahoo.com (F. Sabagh)

ABSTRACT

We analyzed in vitro expansion and differentiation of T progenitor cells from umbilical cord blood in the absence of thymic epithelium. The expansion setup is performed in the presence of SCF, IL-7 , and IL -2 with autologous serum .Using CBMCs as initial source , we compared the growth kinetics of several cell populations in either whole CBMC or CD34+ -enriched-, as well as in CD3CD4CD8-depleted expansion assays by FACS analysis. After 11 days of culture, cell increase values were about 7 fold for CD3+, 6 fold for CD3+CD4+, 7 fold for CD3+CD8+, 4fold for CD3+CD56, 6fold for CD56+, and 0.2 fold for CD34+. We characterized the developmental state of these cell populations by RT –PCR analysis of the lymphoid differentiation markers RAG-1 and pre T-Alpha. In all samples , transcripts of both markers could be detected from day 0 though day 11, however , in case of pre – T-Alpha, nested PCR was always required , indicating lower expression . These findings; therefore, demonstrate that T-cell differentiation events (as opposed to mere expansion) do occur in stroma cell free expansion assays.

Keywords: T cell expansion; Cord Blood; SCF; IL-7; IL-2

INTRODUCTION

Human umbilical cord blood (CB) is an alternative source to marrow for expansion of progenitor cells [1-2]. Mature T cells in CB are mostly resting [3]. The content of CD34+ progenitor cells in CB is approximately 0.4%. The common protocols for expanding progenitor cells into T cells from different sources always require the presence of thymic stroma layer [5-7], e.g. fetal thymic organ culture (FTOC) is a well defined system for the expansion and lymphopoiesis of T cells [8-10]. Generally, for T lymphopoiesis progenitor cells have to migrate through the thymus by expressing specific surface markers such as CD2, CD7, CD3, CD4, CD8, and the T cell receptor (TCR). The chronological order of expressing these surface antigens is briefly from CD3-CD4-CD8- (triple-negative, TN) to low CD4+ and to the double – positive (DP, CD4+ CD8+) stage. These DP cells undergo the positive and negative selection and will be finally single – positive (CD+, or CD8+, SP) with concomitant expression of the TCR. In mouse cells the TN stage is well defined through the alternate expression CD25 and CD44. They express successively CD25-CD44+, CD25+CD44- and finally CD25- and CD44- [11-13]. The branch point of lineage separation (TCRαβ versus TCRγδ positive T cell) is at the TN CD25+CD44- stage. The rearrangements of TCR genes start with transcription of RAG -1 (recombination activating gene) and RAG-2, which synergistically cut the DNA [14]. During this phase the TCRβ and the pre-Tα chains are co-expressed and form the pre TCR [15-17]. From this time of the thymocytes become DP and the thymic selection process begins. Mature SP CD3+TCRαβ +thymocytes do not express pre Tα or RAG-1/2 anymore, and are able to migrate out of the thymus.

So far, all these developmental steps and their regulation have not been completely understood; some developmental events are under control of some cytokines such as IL -7(Interleukin-7) IL-7 is necessary for the activity of RAG proteins

during the recombination of sub-genic elements of the respective TCR chain [18]. More immature T progenitors are CD34+ and express the c-kit (CD117) receptor. These cells can be activated through stem cell factors (SCF) and are than able to undergo further differentiation. Interestingly, activation through IL-2 does not seem to be necessary, because in IL -2 knockout mice mature T cells could be detected [19]. However, IL-2 is discussed as a possible inhibitor of myelopoisis (A.R.M, unpublished results).

Extrathymic differentiation of T cells is shown for intraepithelial lymphocytes (IELs) in the gut [20]. This kind of differentiation produces also mature T cells, but some distinct cell populations such as DN TCRαβ+, CD8αα and TCRγδ T cells show a higher increase [21]. It is not completely known, however, if this kind of T cell development is totally independent from the thymus, since it is conceivable that soluble factors from the thymus can drive the differentiation of these cells [22].

In the present study we demonstrate that T cell expansion with SCF, IL-7, and IL-2 from human umbilical cord blood is a suitable system for generating mature functional T cells. We assume that expansion of T progenitor cells and T cells include both proliferation and T lymphopoiesis. We; therefore, analyzed temporarily expressed genes, required for T cell maturation such as RAG-1 and pre-Tα. Expression of these genes demonstrates that immature T cells are in the developmental phase recombining their TCRs.

MATERIAL AND METHODS

Collection of umbilical cord blood

Cord blood was collected into 150 ml collection bags (MS22204Q; Macopharma, Langen, Germany) containing 21 ml citrate-phosphate dextrose (CPD) as anticoagulant in the Obstetric Departments with the informed consent of the mother .

Isolation of mononuclear cells

Cord blood was sedimented (1500 rpm, 5 min), the serum supernatant was preserved, inactivated (56 C, 30 min) and filtered (0.4 µm sterile filter , Sartorius , Gottingen Germany). The buffy coat was put on a ficoll gradient (Seromed Ficoll Separating Solution, Biochrom, Berlin, Germany), and centrifuged (1800 rpm , 10 min). Later on, the mononuclear cells were isolated and washed twice in PBS (Serag-Wiessner, Naila, Germany). Erythrocyte lysis was done with 5 ml Ammoniumchloride (containing 0,3g NH4Cl, 1g KHCO3, 0.0375g Na-FDTA per liter) and 20 ml PBS for 10 min at 4º C so that the cells were sedimented as described above and re-suspended in PBS. Cell count was performed with a hemocytometer and additionally with the CellDyn 3500 analyzer (Abbott, Wiesbaden, Germany).

Freezing and thawing of cord blood

Pellets form cord blood samples were re-suspended in ice-cold freezing medium (65% Ex vivo 10 (Biowhitaker, Walkersville, USA), 10% Dimethylsulfoxide (Sigma, Deisenhofen, Germany) 25% inactivated and filtered autologous serum, which is diluted 1/10 with CPD as anticoagulant). Thawing of CB samples was performed as described by Rubinstein et al, [23].

Enrichment of CD34+ progenitor cells

CD34+ cells were separated from CB mononuclear cells (according to the instruction of the manufacturer) with the CD 34 /Magnetic. Activated Cell Sorting isolation system (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany), equipped with VS+ separation columns. The purity was analyzed with cytoflourometry after staining with CD34+ antibody.

Depletion of T cells

T cells were depleted with CD3-, CD4-, and CD8- MACS-beads at once with the previously described MACS isolation system using VS-columns. The purity of depletion was quantified by cytofourometry after staining with anti-TCRαβ andtibody.

Cell lines and thymocytes

The immature T cell line Molt -3 was used as a control for RAG-1 gene expression [24]. On the other hand, we used Thymocytes served as control for RAG-1 andpre – Tα expression [25]. Ex vivo expansion of mononuclear cells, CD34+ progenitor cells, and T cell depleted cells.
For expansion, cells were cultured 10-11 days in Ex vivo 10 medium supplemented with 15%

autologous serum. The following factors are added: 10 ng/ml SCF (StemCell Technologies, Vancouver, Canada), 10 ng/ma IL-7 (Genzyme, Russelsheim, Germany), and 100 U/ml IL-2 (proleukin, Chiron, Ferwald, Germany) in 24 well plates (Costar, Cambridge, MA, USA).

Cell counts are preformed at day 0, 4, 8 and 10/11. The initial concentration was 1 ×10 5 for CBMCs and CD3-CD4-CD8- cells and 2 ×10 5 c/ml for the CD34+ cell population.

Analysis of surface markers by flow cytometry

Monoclonal antibodies (MoAbs) specific for the following markers were used: CD3, CD4, CD8, CD34, CD45, CD45RO, CD45RA, CD56, CD117, TCRαβ, TCRγδ were conjugated antibodies directed to CD34, CD45RA, CD4, CD8, CD3, CD45RO, TCRγδwere obtained from Coulter Immunotech (Hamburg, Germany) PE –conjugated antibodies are specific for CD4, CD117, TCRαβ (coulter Immunotech), and CD56 (pharmingen) cychrome conjugate MoAbs to CD45, CD4, CD3 were obtained from Coulter Immunotech, MACS-CD3-Microbeads from Miltenyi Biotech (Bergisch Gladbach, Germany) and were used to swparate cell suspensions . Determination of cell surface antigens was performed on a cell analysis / sorting unit from Coulter, which is equipped with an argon ion laser and a helium neon laser. Three color analyses with cord blood cells were gated on lymphocytes with exclusion of cell debris. Analysis of cells for expression of cell surface antigens was achieved with data acquisition research software (Coulter). The γ1/γ1 IgG control antibody (Becton- Dickinson, Erembodegem – Aalst, Belgium) was used for the same cells and also propidium iodide was added to detect dead cells.

cDNA synthesis and polymerase chain reaction

Total RNA was isolated from 1 ×10 5 cells with the RNeasy Kit according to the manufactures description (Qiagen, Hilden, Germany). About 500 ng of total RNA was added to the cDNA synthesis reaction using the following protocol: 6 µ1 of 5 × buffer (Promega, Madison, USA), 20U of RNAsin (promega, Madison, USA), 100 pmol of random hexamer primers (Pharmacia, Uppsala, Sweden (2 µ1 of dNTP solution (10 mM each, Pharmacia, Uppsala, Sweden) and 9 U AMV Reverse Transcriptase (Promega, Madison, WI, USA) filled up with water to a final reaction volume of 30µ1. The reaction mixture was incubated at 42 C for 45 min. Afterwards, the reaction was terminated by heating 5 min 95º C and then quick cooled on ice. The cDNA was used afterwards for the PCR reaction, or stored at -20° C. PCR reaction mixture: 1 to 10 µ1 cDNA reaction mixture (1 µ1 for GAPDH, 10 µ1 FOR RAG-1, and pre –Tα) 10×PCR buffer (Gibco BRL, Gaithersburg, USA), 0.2 mM of dNTP, 50 pmol of each primer, 2 U of Taq DNA polymerase (Gibco BRL, Gaithersburg, USA). Water was added to 100 µ1 reaction volume.
Amplifications were carried out with a thermal cycler (GeneAmp PCR system 2400, Perkin Elmer, Norwalk, USA). The following profile over 35 cycles was performed: 3 min 94C for the first delay, 30 sec at 94º C for denaturation, 30 sec at 59º C for annealing, 30 sec at 72C for extension, and the last delay at 10 min 72º C .10 µ1 of the PCR reaction mixture were electrophoresed through 2% agarose gels stained with ethidium bromide and visualized under UV light. The following primers were used: RAG15 -5'-GAA CAC ACT TTG CCT TCT CTT TGG-3' RAG-1 3' |5' –GTC CAG CCC TAC CCA CAG GTG T-3 ': pre Tα5':5' –GTC CAG CCC TAC CCA CAG GTG T-3'pre-Tα3':5-CGG GAA TTC GAC GTC CCT GGC TGT AGA AGC CTC TC-3' pre –Tα-nested 5':5-TCT GGC CCC ACC AAT CAT GC-3',Pre-Tα-nested3.'5-TGT ACT CCT GCT GTG AG-3.GAPDH5.5-ACG GAT TTG GTC GTA TTG GGC G-3,GAPDH3.5-CTC CTG GAA GAT GGT GAT GG-.3

Confirmation of PCR products using cycle sequencing with flourophor labeled primers Sequencing was preformed with the sequencing kit according to the protocol of the manufacturer (Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7- deaza-dGTP , Amersham Life Science , Buckinghamshire , England).using flourophor labelled primers (MWG ,Ebersberg,Germany).An infrared fluorescence technology (DNA sequencer model DNA4000L,LI-COR,Lincoln ,USA) was used for the detection of the sequencing products.

RESULTS

Growth kinetics of CB mononuclear cells, CD34+ enriched progenitor cells and T cell depleted cells.

The used expansion system was designed to show *de novo* generation of T lymphocytes and not for the highest increase in cell number. The main criterion for expansion of cells is the general increase in cell densities. The chief characteristic of this study is the production of T helper cells from mononuclear cells. To compare the obtained data with different cell populations for expansion, we used T lineage depleted cells (CD3-CD4-CD8-) and CD34+ progenitor cells to demonstrate any differences and dependency or independence of the specific cell populations form each other. CB-MNC grow from 1×10 5 up 7×10 5 c/ml during 11 days of culture (all mean values, n=8) .In contrast, CB CD34+ cells grow from the initial density 1, 4×10 5 to 2, 6×10 5 c/ml during 10 days of culture (n=2). Depleted cells (CD3-CD4-CD8-cells) don't increase in cell number (n=2), but decrease form 1×10 5 c/ml to 4×10 4 c/ml during 11 days of culture (Figure1).

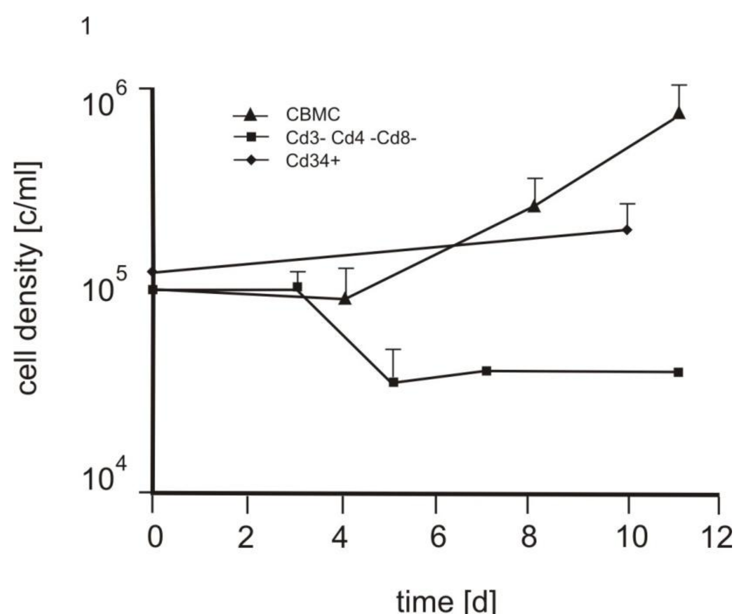


Figure 1. Kinetics of ex v ivo expansion of CBMCs in the presence of SCF, IL-7, and IL-2 over 10 days (CD34+) and 11days (CBMCs, CD3-CD4-CD8-cells). The results of eight (CBMCs) and two (CD3-CD4CD8-and CD34+cells) experiments are expressed as mean ±SD for total cell growth.

Phenotype analysis and growth of *ex vivo* expanded T and NK cells

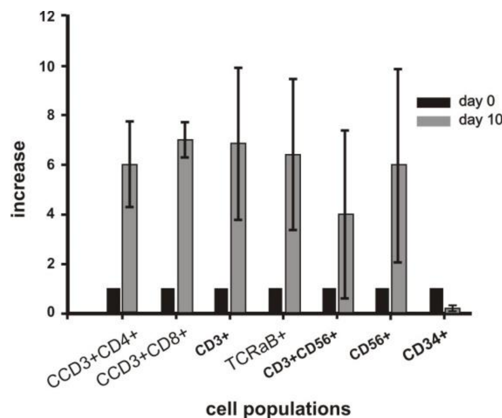
We counted the cells of relevant populations for T and NK cell development, for instance CD3+Cd4+ (T helper cells), CD3+CD8 (cytotoxic T cells), and all T cells which are CD3+, respectively TCRαβ+ (Figure 2). The increase value in CBMC culture for TCRαβ+ cells is 6. 4 fold, T helper cells (CD3+CD4+) increase 6 fold and cytotoxic T cells (CD3+CD8+) increase 7 fold. In general, all CD3+ cells including CD3+ CD56+ cell (4 fold increases) show an increase 6. 9 fold. NK cells (CD56+) which are CD3- increases 6 fold in MNC culture. CD34+ cells, which represent the progentitors for T cells are undergoing decrease to 0.2 fold. Increase values of T helper cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+), all CD3+ cells (mainly mature T cells), TCRαβ+cells (all mature T cells), a subpopulation of NK cells (CD3+CD56+), all NK cells (CD56+), and progenitor cells (mainly CD34+ cells), after expansion of CBMCs. Six experiments with independent donors are expressed as mean ± SD (day 0 is set to 1) (B) Expansion of T cell depleted cells (CD3-CD4-CD8-) after 11 days illustrated as increase values. The main T cells (CD3+TCRαβ+) population was compared with NK cells (CD3+CD56+and CD56+) and progenitor cells (CD34+). The results of two experiments with independent donors are expressed as mean±SD

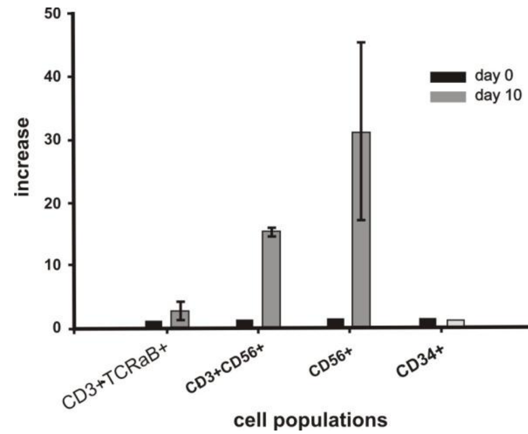
(day 0 is set to 1). (C) Increase of T/NK cell populations from CD34+ enriched cell culture after 10 days in the presence of SCF, IL -7, and IL-2. , Two expansion assays were done with CD34+ cells from independent donors and the increase values are expressed as mean±SD for each cell population (day0 is set to 1) .

To answer the question if this cell population can develop independently from each other, a depletion experiment with CD3+, CD4+, and CD8+ antibodies was performed, which mainly deplete the mature T cell population. Interestingly, this time T cells only show a low increase, e.g. the TCRαβ+cells 1.7 fold on average (Figure 2b). NK cells (all CD56+ cells) show an increase about 30 fold and the CD3+CD56+NK-Subopulation 15 fold.

This finding demonstrates that depletion of CD3+, CD4+ and CD8+ cells efficiently prevent progenitor cells to develop into mature T cells. In contrast, the high increase of NK cells suggests their independent development from T cells and other CD4+ and CD8+ cells.

The CD34+ selected fraction (purity>96%) show a dramatic decrease in CD34 cells (0.01 fold)followed by 10.3 fold increase of CD3+ cells after 10 days of culture (Figure 2c). These results suggest that CD34+progenitor cells differentiate under the described conditions into mature T cells. The CD56+ NK cells expand 6.4 fold, which is nearly identical with the obtained results from CBMCs, however, the CD3+CD56+ NK subpopulation increases only 1.5 fold (CB-MNCs 4fold).





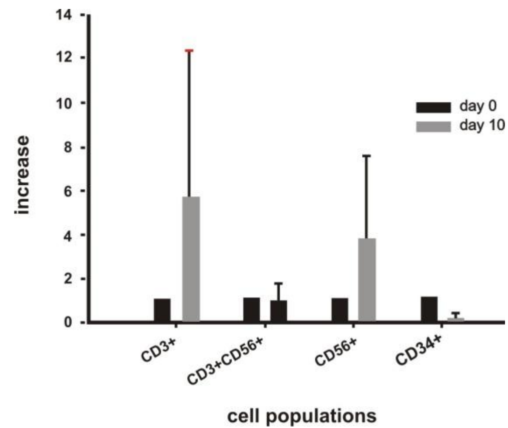


Figure 2. (a) Increased values of mature T Cells, NK cells, Progenitor cells from CBMCs in day 0 and 10/11 **(b)** Expansion of T cells depleted cells after 11 days illustrated as increase value. The comparison of different increase value in T cells, NK cells and progenitor cells on day 0 to 11 **(C)** Increase values of T NK cell culture after 10 days.

Amplification of RAG-1 and pre-Tα transcripts in *ex vivo* culture

To demonstrate the developmental stage of T progenitor cells (conversion of TN to DP cells) a RT-PCR analysis of RAG-1 and pre – Tα transcripts was performed, since mature T cells do not transcribe these temporarily expressed genes. Kinetics of lymphoid gene expression from CBMCs (A and B) after 0, 4, 8, and 11 days of culture and from CD34+ cells (C) after 0 and 10 days in presence of SCF , IL -7, and IL-2. Total RNA was extracted from cells from 4 experiments with independent CB donors, pooled in equal amounts (1µg of total RNA) and subjected to PCR using sequence –specific

primers for RAG-1(A and C,b), and pre Tα (B,b). Five separate replicate PCR reactions were performed for each primer set. Bands of expected size were detected for RAG-1; no amplified transcripts could be visualized for pre-Tα in the first PCR. After the second PCR with nested primer set, the expected amplification products were detected for pre-Tα too. The weak signals for pre –Tα thymus control were intensified by southern blotting with a sequence –specific probe (B,a). For each primer set, a simultaneously run template –free PCR reaction was used as a negative control. Human thymus total RNA (for RAG-1 and pre –Tα) and total RNA from the ALL cell line Molt -3 (only for RAG-1) was used as positive controls to confirm successful PCR reactions. RAG-1 PCR products were also confirmed with southern blotting using sequence –specific primers (C,a). Amplification of GAPDH confirmed the presence of mRNA in all PCR reactions (data not shown) .All PCR products were confirmed by sequencing.

During the 10-11 days of cell expansion culture the half of the cells (from 1 ml culture) were taken for RT-PCR analysis on day 0, 4, 8, and 11, and pooled from 4 independent donors. Monouclear cells from CB show a RAG-1 amplification product at day 0 until 11 at any day of measurement (Figure 3A). This finding indicates the permanent developmental status of the lymphocytes in this *ex vivo* expansion system. GAPDH control shows the same intensity in each sample (data not shown). The pre-Tα transcripts are identified as well also performing nested PCR, which is due to very low expressed status in developing T cell progenitors (Figure 3B).

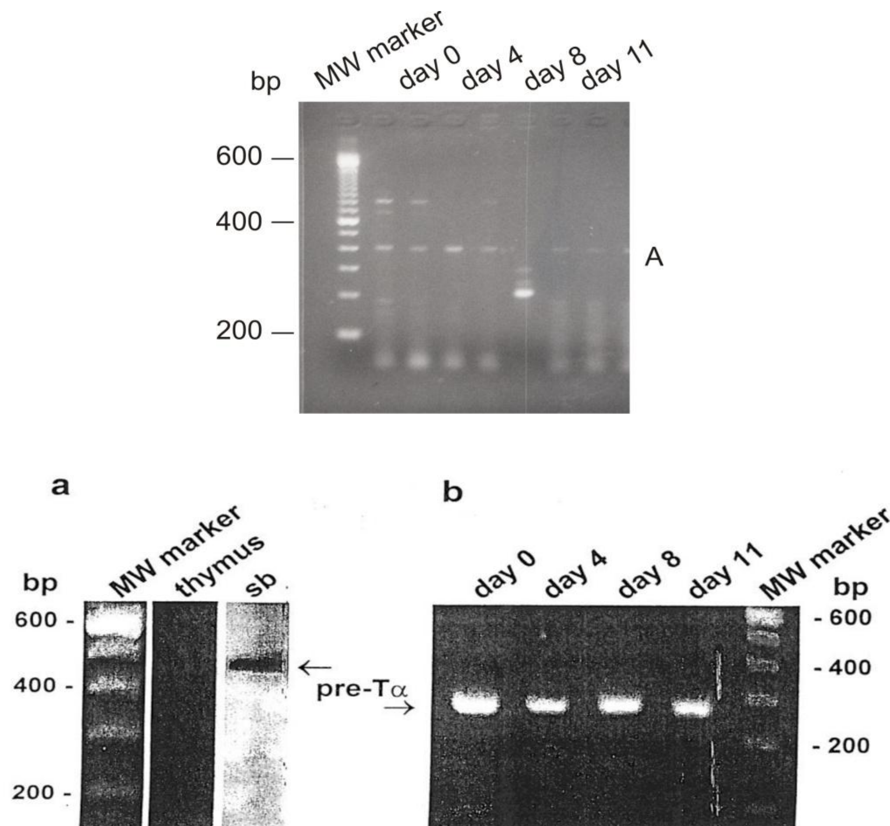


Figure 3. (A) Kinetics of lymphoid gene expression in CBMCs after 0, 4, 8, and 11 days of culture and from CD34+ cells. Total RNA extracted from human thymus to detect RAG-1 in PCR **(a,b)** Detect ion of pr Tα gene from CB(RNA- extract) to perform nested PCR(day to day 11)

DISCUSSION

The present study has demonstrated that T cell expansion in presence of SCF, IL-7 and IL-2 during 10 or 11 days is an efficient way to obtain mature T, and NKcells from CBMCs CD3- CD4-CD8-cells are not or only poorly able to develop into mature T cells in this expansion system, but the capacity to develop into NK cells in the absence of CD3, CD4 and CD8 was not impaired. The main goal in every T cell expansion system is to show the development of T cells (as opposed to mere expansion) which so far has always required the presence of thymic stroma cells such as FTOCs [25-26]. In a postulate extrathymic lymphopoiesis event, it has to be demonstrated that the same genes are temporarily expressed than in thymic development.

Therefore we have analyzed the expression of RAG-1, which is expressed in pre –T and pre –B cells, and pre – Tα, which occurs only in immature developing T cells [27-28]. RAG-1 cuts the genomic DNA for V-(D)-J rearrangements of TCR genes synergistically with RAG-2 and is expressed during the selection of immature T cells, whose auto-reactive potential has been confirmed [29].

In this stage the pre-Tα chain functions as a surrogate for TCRα and these immature cells (mainly DP) undergo further differentiation into SP mature T cells [30]. This differentiation step takes 24-96 h [31]. Our CBMC-expansion assay shows by means of RT-PCR a RAG-1 signal at day 0 which persists until day 11. For this reason it could be assumed that RAG-1 expressing cells that day 0 undergo further differentiation into mature cells and the persisting expression comes from cells, which newly enter the stage of recombining their V(D)J genes. According to this hypothesis CD34+ should have been decreased during this time. It is known that CD34+cells have the potential to differentiate into mature T cells under conditions of thymic microenvironment [32].

Therefore, we analyzed the CD34+ content in CBMCs and in CD34+ expansion assays. In CBMC cultures CD34+ cells decrease 5 fold over 11 days which is supportive for the generation of RAG-1+ cells over the whole period of time. It should be pointed out, however, that the possibility of arrested RAG-1 expressing cells and not be excluded. Separated CD34+ cells (purity>96٪) express a very faint signal for RAG1 at day0, whereas at day 10 the amplification product for RAG-1 is clearly visible (Fig .X). This indicates the differentiation status of cells undergoing lymphopoiesis.

It has been described previously that CD34+ cells are still negative for RAG-1 [33]. However, we suppose that our finding is due to contaminating CD34- cells (approximately 3-4% on average) in the CD34+ preparation. The small amount of RAG-1 mRNA produced by these contaminance could have been amplified with the highly sensitive RT-PCR method.

The total increase of CD3+ cells form enriched CD34+ cells from enriched CD34+ population is 5.6 fold which is comparable to corresponding increase in CBMCs (6.9 fold).

We expected a greater T cell expansion from CD34+ cells than from CBMCs, considering the very low CD34 + cell content is in the CBMC preparation. Our findings, however, may suggest that unlike CBMCs, CD34+ cells require additional cell population or differentiation factors for undergoing lymphopoiesis with the use cytokine combinations. Interestingly, thymocytes which are CD4+CD8+ can undergo conversion into CD4+ or CD8+ T cells without thymic epithelium, however, only in the presence of expressed surface makers like CD2, CD5, CD, CD28,CD49d, CD81, andTSA-1[34]. Cell populations which contain these markers are; therefore, essential for the final step from DP to SP differentiation. Many of these cells were removed during the applied CD3, CD4, and CD8 depletion step. This could be a possible explanation for the absence of the absence of mature SP T cells in T cell depleted culture and for the low increase in T cell markers in CD34+ preparations.

The highest increase for NK and CD3+ NK cells was observed in the CD3-CD4-CD8- population. This finding suggests that the depletion step has removed possible competitors for growth factors. Another explanation could be that after depletion no potential inhibitory signals were available any longer from CD3, CD4, or CD8 carrying cells.

The performed experiments have shown that the used expansion procedure could be useful for the *in vitro* generation of T and NK cells; these generated cells could then be a suitable source

for clinical applications, such as the reconstitution of the T/NK cell repertoire of patients, which are suppressed for immune responses after transplantation, as well as for cell and gene therapy strategies.

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