Detection of a CD4⁺CD8⁻CD3⁻ cell subpopulation during the differentiation of cord blood CD34⁺ cells into T cells *in vitro*

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Abstract

Introduction: Umbilical cord blood contains relatively abundant primitive CD34⁺ hematopoietic progenitor cells which can differentiate into T lymphocytes *ex vivo*.

Materials and Methods: In this study, thymic stromal cells (TSCs) were isolated from aborted fetuses and a monolayer culture system was established. Highly-purified CD34⁺ cells from umbilical cord blood were cultured on the TSCs after limiting-dilution. The cells were then harvested and evaluated for CD4, CD8, and CD3 expression at different time points. CD4⁺CD8⁻CD3⁻ lymphoid progenitor cells that could differentiate into mature T lymphocytes were observed after 15 days when a cocktail of cytokines, including Flt-3 ligand, stem cell factor, interleukin (IL)-12, and IL-2, was added.

Results: These results thus show that CD4⁺CD8⁻CD3⁻ cells can be derived from CD34⁺ cells *in vitro* when cultured on TSCs.

Conclusions: We showed that CD4⁺CD8⁻CD3⁻ cells can be derived from highly purified CD34⁺ cells on TSCs during T-cell lymphopoiesis *in vitro*.

Key words: thymus, cell differentiation, stromal cells, T cells.

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INTRODUCTION

Hematopoietic stem cells (HSCs) are capable of self-renewal and differentiation into erythroid, myeloid, and lymphoid lineages (Mikkola et al. 2003). T-cell lymphopoiesis is largely dependent on the presence of the thymus (Kakinuma et al. 2003; Sugimoto et al. 2002). Generally, T-cell precursors migrate into the thymus from bone marrow and differentiate from CD4⁻CD8⁻ cells into mature CD4 or CD8 single-positive cells via immature CD3⁻CD4⁺ single-positive and CD4⁺CD8⁺ cell stages (Andersson et al. 1994; Guidos et al. 1990). The fate of most developing thymocytes is intrathymic death, either early through a failure of the surface T-cell receptor to be engaged by self MHC (positive selection) or later in a maturation process by which self-reactive thymocytes are eliminated (negative selection). Only 1–2% of total thymocytes are exported to the periphery

after undergoing positive and negative selection (Deftos et al. 2000; Kappler et al. 1987; Kraft et al. 1993; Su and Manley 2002; Swat et al. 1992). CD3⁻CD4⁻CD8⁻ thymocytes result in the sequential appearance of CD3⁻CD4⁺CD8⁻ and CD3⁺CD4⁺CD8⁺ cells, and immature CD3⁻CD4⁺ single-positive cells are thought to be mainly located in the outer cortex and to be blastic and cortisone sensitive (Guidos et al. 1990). Kim et al. (Kim et al. 2005) found that OX40 signals from CD4⁺CD3⁻ cells located in the outer T zone select proliferating Th2 effectors into the memory T cell pool. Lane et al. (Lane et al. 2005) thought that CD4⁺CD3⁻ cells have two functions: organizing lymphoid tissue during development and supporting T-cell help for B cells both during affinity maturation in germinal centers and for memory antibody responses. Their further research showed that CD4⁺CD3⁻ cells act as a tether between the selecting T cells and the follicular dendrit-

ic cells and thus play a role in segregating B and T cells in normal lymphoid tissue (Kim et al. 2007; Lane et al. 2005).

The thymic epithelium is essential for the early T-cell differentiation of hematopoietic precursors as well as the terminal differentiation to mature T cells (Utsuyama et al. 2003). A number of different cell types in the thymus play critical roles in the process of supporting T-cell differentiation, including MHC class II-positive thymic epithelial cells and mesenchymal cells. In general, T-cell differentiation depends on the complex three-dimensional structure of an intact thymic environment. However, it is not clear whether the differentiation from CD34⁺ cells toward T cells can be observed through the immature CD4⁺CD8⁻CD3⁻ cells.

The experimental study of human T-cell lymphopoiesis has been largely limited to the use of the OP9 expressing the Notch ligand Delta-like 1 (OP9-DL1) SCID-hu mouse model (Galy et al. 1993), murine and human thymic organ cultures (Rosenzweig et al. 1994; Yeoman et al. 1993), and human thymic monolayers (Poznansky et al. 2000). Zuniga-Pflucker demonstrated that retrovirally transduced DL1 Notch ligand to the OP9 bone marrow stromal cell line inhibited the development of B cells but favored the development of T cells from fetal liver-derived HSCs and CB-derived HSCs (La Motte-Mohs et al. 2005). In previous studies, thymic stromal cell (TSC) monolayer culture systems provided a useful tool for studying the differentiation of human T lymphocytes. Umbilical cord blood is an alternative source of hematopoietic stem and progenitor cells. In this study we utilized TSC support systems which were based on fetal thymus organ culture as described by Jenkinson et al. (Jenkinson et al. 1982) and Kingston et al. (Kingston et al. 1985) to study T-cell differentiation from CD34⁺ cells purified from umbilical cord blood. The results demonstrated the transient immature CD4⁺CD8⁻CD3⁻ cells could be induced from CD34⁺ cells in the presence of TSCs.

MATERIALS AND METHODS

Thymic organ culture

This study protocol was reviewed and approved by the ethics review committee of our hospital. Human thymus gland was removed from electively aborted fetuses of 18–20 weeks of gestation by aseptic operation in the department of Ob/Gyn of our hospital after the mother's informed consent was obtained. Thymic tissue was processed within 4 h of harvest. The thymic tissue was minced into small fragments, passed through a mesh sieve, and digested into a single-cell suspension by incubation in phosphate-buffered saline (PBS) containing 0.5 mg/ml of collagenase (Sigma, St. Louis, MO, USA) and 2 U/ml DNase I (Sigma) at 37°C for 60 min with frequent agitation. Typically, 1–10×10⁸ cells were obtained per gland, with about 90% viability as assessed by trypan

blue exclusion. The cell suspension was then washed twice in culture media (IMDM; Gibco, Grand Island, NY, USA) containing 10% heat-inactivated human AB serum (HABS) and glutamine (1 mM). Thymic stromal cultures were established using fresh cells in 24-well plates at a concentration of 10⁶ cells per well in 2 ml of medium at 37°C in a humidified atmosphere of 5% CO₂/95% air. After two day's culture, non-adherent cells were removed by washing twice with fresh medium. The monolayer was then incubated in culture media that was changed at least twice a week. Adherent cells were detached by trypsin-EDTA (Gibco, Grand Island, NY, USA) after they reached confluence and seeded at 10⁵ cells/well in 48-well plates (Costar, Cambridge, MA, USA) with IMDM containing 10% HABS and glutamine (1 mM). The second-passage TSCs were used as a feeder layer in the T-cell lymphopoiesis assay.

Purification of cord blood CD34⁺ cells

Umbilical cord blood was obtained from the Department of Ob/Gyn at our hospital after the mother's informed consent was obtained. Cord blood (50–100 ml) was collected in sterile bags containing 28 ml of citrate phosphate dextrose (Sigma) as anticoagulant, brought to the laboratory at room temperature, and processed within 6 h. Mononuclear cells (MNCs) were separated by density gradient centrifugation at 1300 rpm for 15 min at room temperature over Ficoll-Paque (Pharmacia Biotech Inc., Piscataway, NJ, USA). Interface cells were washed twice by mixing with 2 ml of buffer-PBS and centrifuging at 1500 rpm for 8 min at room temperature. The MNCs were resuspended in complete medium and cultured overnight at 37°C to remove adherent cells. Erythrocytes were removed by lysis with 0.15 M ammonium chloride.

Highly purified CD34⁺ cells were enriched from MNCs with two rounds of purification. First, MNCs were incubated with magnetic beads coated with anti-human CD34 antibody (QBEND/10, MACS, Bergisch Gladbach, Germany), then CD34⁻ cells were removed by washing three times with buffer-PBS in MACS. The highly purified CD34⁺ cells were collected according to the protocol described by the manufacturer. The collected CD34⁺ cells were again passed through another column to achieve a higher purity to ensure that the cells added to the TSCs were derived from CD34⁺ cells, not CD34⁻ cells. The human CD34⁺ cells purified from cord blood MNCs using Mini-MACS were further depleted of cells expressing the lymphoid markers CD2 or CD3 to minimize T-cell contamination.

Limiting dilution and T-cell lymphopoiesis assay

Immature and mature T cells were generated from the CD34⁺ cells using the previously established method of re-seeding subconfluent cultures (Rosenzweig et al. 2001). The purified CD34⁺ cells were plated on the adherent TSCs by limiting-dilution analysis, which is

used to estimate HSC activity. Four dilutions were used, with three replicate wells for each. A minimum of 50 CD34⁺ cells per well were plated in 1 ml of IMDM containing 10% HABS, 10 ng/ml recombinant human interleukin-12 (rhIL-12; Perpertech, USA), 30 ng/ml recombinant human Flt-3 ligand (rhFL; Perpertech, USA), 50 ng/ml recombinant human stem cell factor (rhSCF; Perpertech, USA), and 50 IU/ml rhIL-2 (Perpertech, USA) at 37°C in 5% CO₂/95% air. The dilutions were chosen to be maximally informative after two weeks of incubation. The cytokine combination has been shown to promote CD34⁺ cell expansion and T-cell differentiation (Mourcin et al. 2005; Nelson and Willerford 1998). Half of the medium in each well was removed and replaced with fresh medium containing cytokines every 3–4 days. Immunophenotyping of the cultured cells was carried out with flow cytometry (XL-AD 2517, Beckman-coulter).

The T-cell subpopulation was detected by staining with fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (mAb) against CD4 (CD4 FITC, BD Pharmingen), phycoerythrin (PE)-labeled mAb against CD8 (CD8 PE, BD Pharmingen), and phycoerythrin-cyanine 5 (PC5)-labeled mAb against CD3 (CD3 PC5, BC Pharmingen) and analyzed by flow cytometry at days 15, 18, 21, 24, 27, and 30 during the culture period. After being harvested by gentle aspiration and washed twice in PBS, the cells were stained in a final volume of 100 μ l with the above antibodies. The isotype controls used

were IgG1-FITC, IgG1-PE, and IgG1-PerCP, respectively. The stained samples were washed twice in PBS and flow cytometry analysis was performed immediately. System II Software or Cofit software was used for data analysis.

TSCs without CD34⁺ cells were cultured at the same time and harvested using trypsin-EDTA at day 0 and day 15. These cells were stained with the same procedure and served as controls for the T-cell lymphopoiesis assay.

Statistical analysis

Comparisons were made using standard statistical tests. Results are presented as the mean $\pm$ standard deviation (SD). A p value <0.05 was considered significant differentiation. CD4⁺CD8⁻CD3⁻ cells were compared with other subsets using repeated measures analyses of variance. Statistical analyses were performed using CHISS (Chinese High Intellectualized Statistical Software) version 2004.

RESULTS

As reported by others (Sarun et al. 1998), TSCs from human thymus were isolated as a reliable feeder layer for T-cell lymphopoiesis *in vitro*. The TSCs were monitored daily. The cell clones could be seen on day 3 and

Fig. 1. Phenotype analysis of TSCs. TSCs without CD34⁺ cord blood cells were detached by trypsin-EDTA at day 0 and day 15. Cells were stained with CD3 PC5, CD4 FITC, and CD8 PE mAbs. The result showed that the TSCs were negative for CD4, CD8, and CD3, thus excluding the possible contamination of CD3⁻CD4⁺CD8⁻ from TSCs.

became confluent on day 8. The TSCs were composed mainly of epithelial cells (60–90%). A typical TSC looked like a fibroblast. Flow cytometry analysis showed that the TSCs were negative for CD3, CD4, and CD8 (Fig. 1).

About $3\text{--}6 \times 10^5$ CD34⁺ cells were isolated from each cord blood unit and the purity of the isolated CD34⁺ cells ranged from 95 to 99%. When the cord blood CD34⁺ cells were cultured on a TSC layer in medium supplemented with IL-12, FL, SCF, and IL-2, they underwent T-cell lymphopoiesis *in vitro* in a manner that recapitulated T-cell ontogeny *in vivo*. The cell number increased rapidly. The cells were harvested at the indicated time points for phenotype analysis. After a two-week culture period, mainly CD4⁺CD8⁺ (double-positive) immature T lymphocytes were detected. At the same time we found a small immature CD4⁺CD8⁺CD3⁺ cell subset ($3.78 \pm 0.49\%$) (Fig. 2). Then the proportion of immature CD4⁺CD8⁺ T lymphocytes decreased with time, coincident with a gradual increase in mature T lymphocytes (Fig. 3). To confirm that the immature and mature T cells were derived from cord blood CD34⁺ cells, we harvested and stained control TSCs at dif-

ferent time points and could not detect any T cells (either immature or mature) by flow cytometry analysis.

DISCUSSION

TSCs have been proven to be a valuable model in the study of sequential differentiation of murine, chicken, rhesus, and human thymocytes. In this study we observed that the highly purified cord blood CD34⁺ cells differentiated into T cells on TSCs after limiting dilution following a pattern that mirrors T-cell development *in vivo*. The T cells derived from this culture system have the phenotype of mature T cells. We can exclude the possibility that these T cells are residue T cells in the cord blood or arise from the TSCs based on the following facts. Firstly, two rounds of MACS were performed for the enrichment of CD34⁺ cells. Flow cytometry analysis confirmed that the purity of the CD34 cells was higher than 99% and residue T cells were less than 1%. Secondly, the number of CD34⁺ cells in each well was controlled by limiting dilution. As

Fig. 2. T-lymphocytic phenotype analysis at different time points show T-cell lymphopoiesis *in vitro* in a manner that recapitulates T-cell ontogeny *in vivo*.

Fig. 3. Differentiation of CD34⁺ cord blood cells. During 21 days of culture, CD34⁺ cells differentiated mainly into immature CD4⁺CD8⁺ T lymphocytes. At the same time we found a small immature CD3⁻CD4⁺CD8⁻ cell subset ($3.78 \pm 0.49\%$). Then the proportion of CD4⁺CD8⁺ and CD3⁻CD4⁺CD8⁻ immature T lymphocytes decreased with time, coincident with a gradual increase in mature T lymphocytes.

described above, the enriched CD34⁺ cells contained almost no CD34⁻ cells. Thirdly, we could not find any T cells in the control TSC-only culture, thus excluding the possibility that the immature and mature T cells might originate from residual mature thymocytes in the TSCs. Most importantly, this experiment was repeated a total of five times by different researchers and the same result was achieved each time.

It was previously reported that T-cell precursors migrating from the bone marrow can differentiate from CD4⁺CD8⁻ cells into mature CD4⁺ or CD8⁺ cells via immature CD3⁻CD4⁺ and CD4⁺8⁺ cell stages (Andersson et al. 1994). Egerton et al. (Egerton et al. 1990) found that many of the mature thymocytes derive from a small non-dividing cortical thymocyte pool (CD4⁺CD8⁻CD3⁻), rather than the earliest dividing CD4⁺CD8⁺ blasts, by labeling dividing thymocytes and their progeny *in vivo* with [³H]-thymidine. They showed that one-for-one differentiation from cortical cells by CD4⁺CD8⁻CD3⁻ cells was as important as selective clonal expansion during T-cell lymphopoiesis. Nanki et al. (Nanki et al. 1998) used the PCR-ELISA technique to examine immature CD4⁺CD8⁻CD3⁻ T lymphocytes for their BJ gene repertoires and found the overall BJ gene repertoire was developed before antigenic selection, which was then further modified by thymic selection and peripheral stimulation.

Our results also demonstrated that human CD4⁺CD8⁻CD3⁻ cells derived from CD34⁺ cells *in vitro* may be an immature subset during T-cell lymphopoiesis. Kraft et al. (Kraft et al. 1993) described this CD4⁺CD8⁻CD3⁻ cell population, which represents an alternative intrathymic T-cell precursor, as a monocytic population. Gardner et al. (Gardner et al. 1998) also found that after two weeks of culture on a thymic monolayer, donor-derived cells maintained the classic phenotypes of cortical thymocytes (CD4⁺CD8⁻CD3⁻ and CD4⁺CD8⁺) in proportions similar to those of fresh

thymic samples. Kelly and Scollay (Kelly and Scollay 1992) examined cells in the peripheral lymph nodes during the first week of life to study mature T cells migrating from the thymus to peripheral lymphoid organs for their phenotype and function and they found a high proportion of CD4⁺CD3⁻ cells that diminished rapidly in proportion to the number of CD3⁺ cells increasing after birth. However, they could not determine their origin and function. Our study provides new evidence that these CD4⁺CD8⁻CD3⁻ cells may arise from CD34⁺ cells.

Our study demonstrated the kinetics of CD4⁺CD8⁻CD3⁻ cells, CD4⁺CD8⁺CD3⁺ cells, and CD4/8 single-positive cells during 15 days in culture. These results suggest that the immature CD4⁺CD8⁻CD3⁻ cells and CD4⁺CD8⁺CD3⁺ cells develop differently. As Kraft et al. (Kraft et al. 1993) proposed, the CD4⁺CD8⁻CD3⁻ population is a sequential intermediate between CD3⁻CD4⁺CD8⁻ and CD4⁺CD8⁺CD3⁺. Our result support Kraft's proposal as we observed that the proportion of the CD4⁺CD8⁻CD3⁻ subset decreased with time while the proportions of the CD3⁺CD4⁺ or CD3⁺CD8⁺ subsets increased.

The functions of CD4⁺CD8⁻CD3⁻ cells are still unclear, but it is obvious that they are clinically important. Sugimoto et al. (Sugimoto et al. 2002) reported a woman who was diagnosed with monoclonal CD3⁻CD4⁺-associated idiopathic hypereosinophilic syndrome, and hydroxyurea lessened only the number of peripheral eosinophils, but not the number of clonal CD3⁻CD4⁺ T lymphocytes. Brugnani et al. (Brugnani et al. 1997) found that purified CD3⁻CD4⁺ cells showed a Th2/Th0 pattern of cytokine production after activation, secreting high levels of IL-5 and IL-4 but a low level of interferon- γ . Nasu et al. (Nasu et al. 1994) reported a patient with adult T-cell leukemia in the smoldering stage with an aberrant phenotype of CD3⁻CD4⁺CD8⁻ and the phenotype of these cells transformed to CD3⁺CD4⁺CD8⁺ after one month as the disease progressed. Estes et al. (Estes et al. 1993) demonstrated that CD3⁻CD4⁺ cells may be related to the stress response of the parasite expanded in the spleens and livers of *M. corti*-infected animals. Kim et al. (Kim et al. 2006) found that CD4⁺CD3⁻ accessory cells are distinct from dendritic cells, T cells, and other lymphocytes by genetic fingerprint, and neonatal CD4⁺CD3⁻ cells up-regulate both CD30L and OX40L after adoptive transfer into an adult recipient. Their further study show that CD4⁺CD3⁻ accessory cells guide the migration of B and T cells to their respective locations by activating stromal cells to up-regulate CCR7 and CXCR5 ligands and are capable of the segregation of lymphocytes into B- and T-cell areas (Kim et al. 2007).

In conclusion, we showed that CD4⁺CD8⁻CD3⁻ cells can be derived from highly purified CD34⁺ cells on TSCs during T-cell lymphopoiesis *in vitro*. The characteristics and function of this cell subpopulation deserve further study.

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REFERENCES

- Andersson G, Owen JJ, Moore NC et al (1994) Thymic epithelial cells provide unique signals for positive selection of CD4⁺CD8⁺ thymocytes in vitro. *J Exp Med* 179:2027–2031
- Brugnoni D, Airò P, Tosoni C et al (1997) CD3–CD4⁺ cells with a Th2-like pattern of cytokine production in the peripheral blood of a patient with cutaneous T cell lymphoma. *Leukemia* 11:1983–1985
- Deftos ML, Huang E, Ojala EW et al (2000) Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. *Immunity* 13:73–84
- Egerton M, Scollay R, Shortman K (1990) Kinetics of mature T-cell development in the thymus. *Proc Natl Acad Sci USA* 87:2579–2582
- Estes DM, Turaga PS, Sievers KM et al (1993) Characterization of an unusual cell type (CD4⁺CD3[–]) expanded by helminth infection and related to the parasite stress response. *J Immunol* 150:1846–1856
- Galy A, Verma S, Barcena A et al (1993) Precursors of CD3⁺CD4⁺CD8⁺ cells in the human thymus are defined by expression of CD34. Delineation of early events in human thymic development. *J Exp Med* 178:391–401
- Gardner JP, Rosenzweig M, Marks DF (1998) T-lymphopoietic capacity of cord blood-derived CD34⁺ progenitor cells. *Exp Hematol* 26:991–999
- Guidos CJ, Danska JS, Fathman CG et al (1990) T cell receptor-mediated negative selection of autoreactive T lymphocyte precursors occurs after commitment to the CD4 or CD8 lineages. *J Exp Med* 172:835–845
- Jenkinson EJ, Franchi LL, Kingston R et al (1982) Effect of deoxyguanosine on lymphopoiesis in the developing thymus rudiment in vitro: Application in the production of chimeric thymus rudiments. *Eur J Immunol* 12:583–587
- Kakinuma S, Tanaka Y, Chinzei R et al (2003) Human umbilical cord blood as a source of transplantable hepatic progenitor cells. *Stem Cells* 21:217–227
- Kappler JW, Roehm N, Marrack P (1987) T cell tolerance by clonal elimination in the thymus. *Cell* 49:273–280
- Kelly KA, Scollay R (1992) Seeding of neonatal lymph nodes by T cells and identification of a novel population of CD3–CD4⁺ cells. *Eur J Immunol* 22:329–334
- Kim MY, Bekiaris V, McConnell FM et al (2005) OX40 signals during priming on dendritic cells inhibit CD4 T cell proliferation: IL-4 switches off OX40 signals enabling rapid proliferation of Th2 effectors. *J Immunol* 174:1433–1437
- Kim MY, McConnell FM, Gaspal FM et al (2007) Function of CD4⁺CD3[–] cells in relation to B- and T-zone stroma in spleen. *Blood* 109:1602–1610
- Kim MY, Toellner KM, White A et al (2006) Neonatal and adult CD4⁺CD3[–] cells share similar gene expression profile, and neonatal cells up-regulate OX40 ligand in response to TL1A (TNFSF15). *J Immunol* 177:3074–3081
- Kingston R, Jenkinson EJ, Owen JJ (1985) A single stem cell can recolonize an embryonic thymus, producing phenotypically distinct T-cell populations. *Nature* 317:811–813
- Kraft DL, Weissman IL, Waller EK (1993) Differentiation of CD3–4–8– human fetal thymocytes in vivo: characterization of a CD3–4–8– intermediate. *J Exp Med* 178:265–277
- La Motte-Mohs RN, Herer E, Zúñiga-Pflücker JC (2005) Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. *Blood* 105:1431–1439
- Lane PJ, Gaspal FM, Kim MY (2005) Two sides of a cellular coin: CD4⁺CD3[–] cells regulate memory responses and lymph-node organization. *Nat Rev Immunol* 5:655–660
- Mikkola HK, Klintman J, Yang H et al (2003) Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature* 421:547–551
- Mourcin F, Grenier N, Mayol JF et al (2005) Mesenchymal stem cells support expansion of in vitro irradiated CD34⁺ cells in the presence of SCF, FLT3 ligand, TPO and IL-3: potential application to autologous cell therapy in accidentally irradiated victims. *Radiat Res* 164:1–9
- Nanki T, Kohsaka H, Miyasaka N (1998) Development of human peripheral TCRBJ gene repertoire. *J Immunol* 161:228–233
- Nasu K, Matsubayashi H, Maeda Y et al (1994) A patient with adult T cell leukemia in smoldering stage expressing an aberrant phenotype of CD3[–] and CD4⁺. *Rinsho Ketsueki* 35:774–779
- Nelson BH, Willerford DM (1998) Biology of the interleukin-2 receptor. *Adv Immunol* 70:1–81
- Poznansky MC, Evans RH, Foxall RB et al (2000) Efficient generation of human cells from a tissue-engineered thymic organoid. *Nat Biotechnol* 18:729–734
- Rosenzweig M, Bunting EM, Damico RL et al (1994) Human neonatal thymic organ culture: an ex vivo model of thymocyte ontogeny and HIV-1 infection. *Pathobiology* 62:245–251
- Rosenzweig M, Yamada K, Harper DM et al (2001) T-cell differentiation of human and non-human primate CD34⁺ hematopoietic progenitor cells using porcine thymic stroma. *Xenotransplantation* 8:185–192
- Sarun S, Dalloul AH, Laurent C et al (1998) Human CD34⁺ thymocyte maturation: pre-T and NK cell differentiation in neonatal thymic stromal cell culture. *Cell Immunol* 190:23–32
- Su DM, Manley NR (2002) Stage-specific changes in fetal thymocyte proliferation during the CD4[–] to CD4⁺ transition in wild type, *Rag1*^{–/–}, and *Hoxa3*, *Pax1* mutant mice. *BMC Immunol* 3:12
- Sugimoto K, Tamayose K, Sasaki M et al (2002) More than 13 years of hypereosinophilia associated with clonal CD3–CD4⁺ lymphocytosis of TH2/TH0 type. *Int J Hematol* 75:281–284

Swat W, Dessing M, Baron A et al (1992) Phenotypic changes accompanying positive selection of CD4⁺CD8⁺ thymocytes. *Eur J Immunol* 22:2367–2372

Tse W, Laughlin MJ (2005) Umbilical cord blood transplantation: a new alternative option. *Hematology Am Soc Hematol Educ Program* 2005:377–383

Utsuyama M, Shiraishi J, Takahashi H et al (2003) Glia maturation factor produced by thymic epithelial cells plays a role in T cell differentiation in the thymic microenvironment. *Int Immunol* 15:557–564

Yeoman H, Gress RE, Bare CV et al (1993) Human bone marrow and umbilical cord blood cells generate CD4⁺ and CD8⁺ single-positive T cells in murine fetal thymus organ culture. *Proc Nat Acad Sci USA* 90:10778–10782