

Key immune cell cytokines have a significant role in the expansion of CD26 population of cord blood mononuclear cells

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Abstract

Background: Umbilical cord blood expresses cluster of differentiation (CD) 26, a fraction of CD34+ cells, negatively regulating *in vivo* homing and engraftment of hematopoietic stem cells. CD26 is highly expressed in various cells such as HSCs, immune cells, fibroblasts, and epithelial cells. It has been shown that inhibition of the CD26 on CD34+ cells improve the efficiency of transplantation of hematopoietic stem and progenitor cells. This study aimed to investigate the effect of key immune cell cytokines on CD26 expansion. **Material and methods:** Cord blood mononuclear cells were cultured for 21 days using the stem cell factor, fetal liver tyrosine kinase 3 (Flt3) ligand (FL), interleukin (IL) 2, IL7, and IL15. Harvested cells were analyzed by flow cytometry at distinct time points. **Results:** Our results showed that utilization of IL7 significantly improved the expression of total CD26+ cells (8.6-fold higher). When either IL2 or IL15 were added to the culture, the expression also improved 2.5-fold. The IL2 and IL7 showed significant effect on the expansion of both the CD26+ and CD26– fractions of the CD34+ cells. However, the effects of IL15 on CD26+ and CD26– expansion were similar. **Conclusion:** Taken together, our data suggested that using IL7 causes higher proliferation of CD26 cells in comparison to that seen under other culture conditions.

Keywords: cytokine, CD26 cells, CD34 cell, umbilical cord blood

Introduction

Umbilical cord blood (UCB) has been used as an alternative source of hematopoietic stem and progenitor cells (HSC/HPCs) for transplantation (Christopherson et al. 2007) in hematologic malignancies and non-malignant disorders (Delaney et al. 2010, Christopherson et al. 2007). UCB has low histocompatibility and low risk of graft-versus-host disease (GVHD) (Wagner et al. 1995, 2002). In UCB transplantation, the CD26+ subset that is a fraction of CD34+ cells,

negatively regulates *in vivo* homing and engraftment of HSCs (Christopherson et al. 2004). CD26 is highly expressed in various cells such as hematopoietic stem cells, immune cells including T, B, and NK cells, fibroblasts, and epithelial cells (Drucker 2007, Vanham et al. 1993, Kahne et al. 1999). CD26 is a dipeptidase that cleaves cytokines from the N-terminus of peptides (Christopherson et al. 2002). The CD26 marker plays an important role in cell-cell interactions, cell adhesion, apoptosis, immune cell regulation, and cancer cell migration (Yu et al. 2006, Christopherson et al. 2012). It also regulates the trafficking of CD34+ HSCs, pre-B lymphocytes, and T lymphocytes from bone marrow (Delaney et al. 2010). Studies have reported that inhibition of the CD26 activity on CD34+ cells can improve HSC/HPC transplantation efficiency (Christopherson et al. 2003, 2007, 2012, Campbell et al. 2007, Eric-Nikolic et al. 2011).

Due to the limited number of cord blood cells collected, many laboratories carry out *ex vivo* stem cell expansion to make UCB more suitable for transplantation (Rao et al. 2012, Xu and Reems 2001). However, for clinical application, the expansion of cord blood HSCs is possible by using some extrinsic regulatory factors including hematopoietic cytokines (a group of glycoproteins). Cytokines are produced by many types of hematopoietic and non-hematopoietic cells (Mayani et al. 1993). For example, stem cell factor (SCF) and Flt3 ligand (FL), have been used as key cytokines for HSC/HPC proliferation (Ueda et al. 2000). It has been shown that SCF improves homing and proliferation of UCB cells in pre-clinical models (Ueda et al. 2000, Boelens et al. 2013). The FL is involved in short-term expansion and also plays a role in the proliferation and differentiation of HSC and HPC (Hofmeister et al. 2007). Previous studies have shown that IL7 is crucial for B cell development and the induction of NK cell differentiation (Parrish et al. 2009, Cavazzana-Calvo et al. 1996). IL15 has a mutual role in the regulation of NK cell differentiation and activation (Cavazzana-Calvo et al. 1996,

Vitale et al. 1996, Cheng et al. 2009). Although IL2 is a T cell growth factor, it mediates the activated B cell proliferation and NK cell differentiation (Waldmann 2006, Meazza et al. 2011, Carayol et al. 1998).

In this study, we evaluated the role of IL2, IL7, and IL15 on the CD26 + fraction of cord blood mononuclear cells, and we also assessed the influence of cytokines on the expansion of CD26 + as a subset of CD34 + mononuclear cord blood cells.

Materials and methods

Cell isolation

Cord blood samples were collected from full-term normal deliveries, and diluted at a ratio of 2:1 with phosphate-buffered saline (PBS; SIGMA). Subsequently, mononuclear cells were isolated by centrifugation on Ficoll-Paque (GE healthcare, 1.078 g/ml) at 850 *gm* for 25 min. The mononuclear cells were collected, washed twice in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), and resuspended in RPMI1640 supplemented with FBS, either for culture or for freezing.

Cell culture and culture condition

A quantity of 10^5 cord blood mononuclear cells were seeded in 96-well plates in 250 μ L of RPMI1640 plus Gluta Max 1-time medium (Gibco) containing 20% FBS (Gibco), 1% penicillin/streptomycin (Gibco), and supplemented with cytokines in final concentrations: SCF (40 ng/ml), FL (40 ng/mL), interleukin-7 (IL7, 40 ng/mL), IL15 (40 ng/mL), and IL2 (40 ng/mL) (all cytokines were purchased from PeproTech). Cells were cultured at 37°C for 21 days, and half of the co-culture medium was replaced weekly. On indicated days, cells were harvested and analyzed by FACS to assess the CD26 and CD34 + cells.

Monoclonal antibodies and flow cytometry

Monoclonal antibodies (conjugated with different fluorochromes) for staining of cell-surface antigens were obtained from Abcam for CD34 (581; Abcam) and from Bioscience for CD26 (M-A261; BD Biosciences). Cultured cells were evaluated by flow cytometric analysis every week. Propidium iodide (1.0 mg/mL; Invitrogen) was used to exclude dead cells from the analysis. Cells were analyzed by BD caliber (BD ebioscience), between 10,000 and 30,000 events were collected, and analyses were performed by using flowing software (Perttu Terho, version 2.5.1.).

Statistical analysis

All data were summarized and expressed as mean (SD). The difference between groups was tested by using the independent T test and one-way Analysis of Variance (ANOVA). *P* values < 0.05 were considered to be statistically significant.

This study, considering its experimental issues, was approved by Ethical committee of the Tabriz University of Medical Sciences.

Results

Effect of IL7 on CD26 + cell expansion derived from cord blood mononuclear cells

The important role of IL7 during NK cell development (Vosshenrich et al. 2005) has been previously shown, along with the complementary role of IL7 during B and T lymphopoiesis, similar to other cytokines of the tyrosine kinase family (Sitnicka et al. 2003, 2007).

It has been shown that administration of IL7 improves immune reconstitution without GVHD induction after allogeneic bone marrow transplantation (Alpdogan et al. 2001). Moreover, the CD26 + population has a negative effect on the engraftment of the cord blood CD34 + cell transplantation (Campbell et al. 2007). It is unclear whether IL7 has a significant effect on the expansion of CD26 + cell-derived cord blood mononuclear cells. To evaluate the effect of various cytokines with different combinations on CD26 expansion, we cultured 1×10^5 cord blood mononuclear cells for 21 days in the presence of different combinations of SCF, FL, IL2, IL7, and IL15. Harvested cells were evaluated by FACS at distinct time points (Figure 1B). In the presence of SCF and FL, cultured cord blood mononuclear cells expressed 3% of CD26 + cells in comparison to that in the absence of cytokines, on day 14 (Figure 1C). Moreover, their expression did not significantly increase by adding IL15 (6%) and IL2 (12%). On the other hand, adding IL7 significantly increased the expression of CD26 + cells (26%), (8.6-fold, compared with the culture condition without IL7), and it was also 2.5 times more than that when either IL2 or IL15 were added to the culture (Figure 1C). When a combination of IL7, IL2, and IL15 cytokines were used along with SCF and FL, the expression of CD26 + cells was 23% (Figure 1C). The same results have been obtained with a small change on day 21 (data not shown). These data suggest the significant role of IL7 on the expansion of CD26 + cells derived from cord blood mononuclear cells.

Role of cytokines in generation of CD26 + cord blood mononuclear cells

Previous studies have shown the crucial role of IL7 for B cell development and IL2 induction in NK cell differentiation (Parrish et al. 2009, Cavazzana-Calvo et al. 1996) and T cell proliferation, and also a mediatory role in B cell proliferation (Meazza et al. 2011, Carayol et al. 1998, Waldmann 2006). Furthermore, IL15 has a significant role in the regulation of NK cell differentiation from CD34 + progenitor cells (Cavazzana-Calvo et al. 1996, Vitale et al. 1996, Cheng et al. 2009). Using the combination of cytokines is an alternative option to increase the survival, differentiation, and proliferation of cord blood cells in transplantation (Mayani et al. 1993, Bordeaux-Rego et al. 2010). Regarding the influence of IL2, IL7, and IL15 on the expansion of the CD26 + subset, cord blood mononuclear cells (1×10^5) were cultured in the presence of IL2, IL7, and IL15 in 96-well plates, and the harvested cells were evaluated by FACS on days 0, 7, 14 and 21. The findings revealed that CD26+ -derived cells had increased in the culture condition of SCF and FL, and the results were similar in both cases of addition of IL15, or of a combination of IL2,

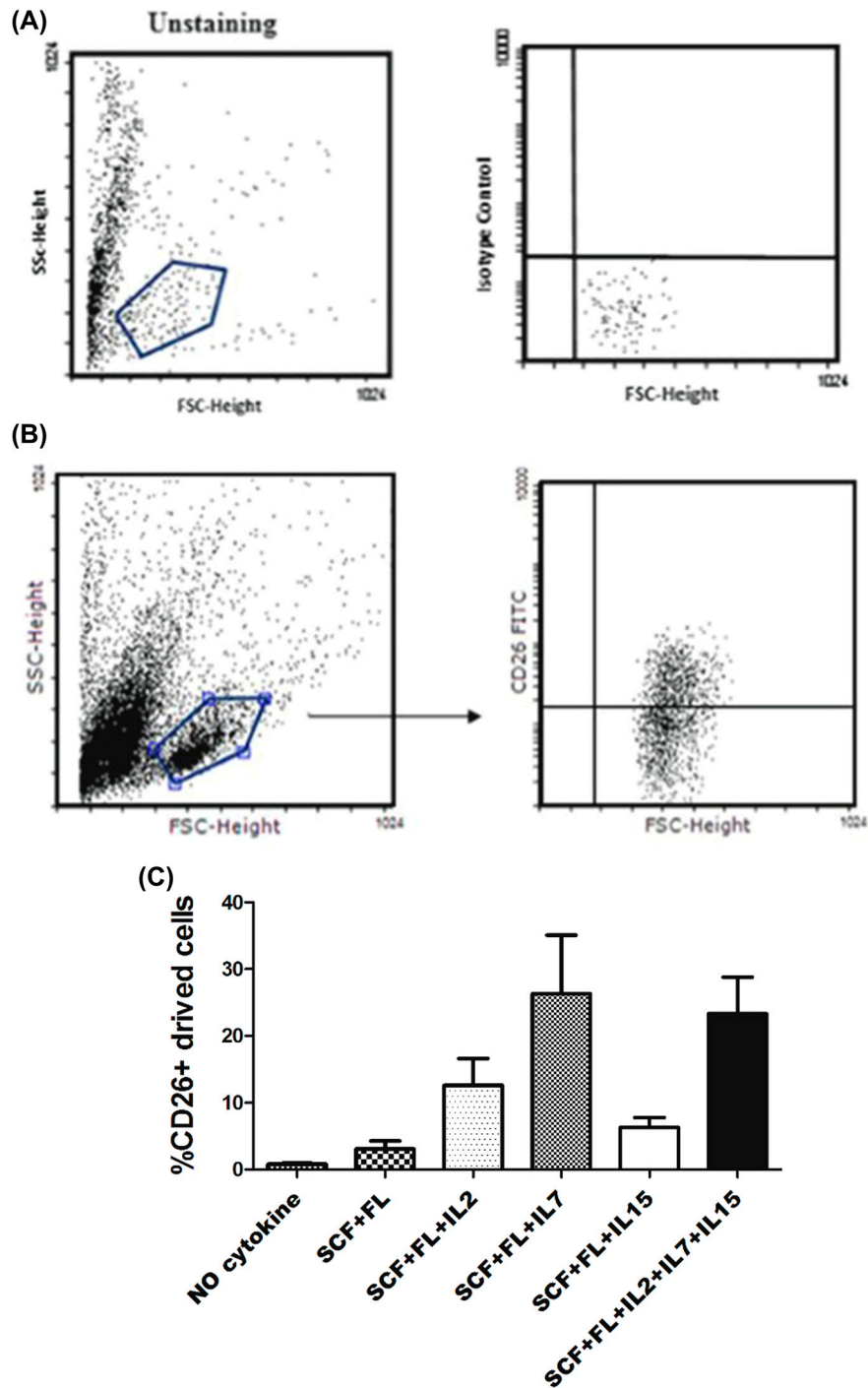


Figure 1. IL7 Ligand generates increased CD26 + cells from cord blood mononuclear cells. 10^5 cord blood mononuclear cells cultured on 96 well plate by using different combination of cytokines and CD26 + cells analyzed by FACS in day 7, 14, 21. (A) isotype control sample. (B) Representative FACS profiles from mononuclear cord blood cultured cells. (C) Mean (SD) proportion of CD26 + cells after 14 days; $p < 0.001$.

IL7, and IL15 (30–60%) (Figure 2A, B, D). In the presence of cytokines SCF, FL, and IL2, the percentage of CD26 reached 11% on day 14, but it declined to 7.8% on day 21 (Figure 2C). The CD26 + cells dramatically increased with a combination of SCF, FL, and IL7, from day 0 to day 14, and remained at the same level on day 21 (30%) (Figure 2E). Taken together, the CD26 + fraction increased with IL7 and IL2, but on day 21, it remained constant for IL7 and reduced for IL2.

It has been shown that mean fluorescence intensity (MFI) of the CD26 + population in lymphoid cells is low in

breast cancer (Eric-Nikolic et al. 2011). Moreover, previous studies have reported that the MFI and the percentage of CD26 + cells in the PHA-stimulated PBMC or lymphoblasts increased in the presence of IL2 (Cordero et al. 1997, Salgado et al. 2000). Considering the influence of key immune cell cytokines on the CD26 + fraction on the cord blood mononuclear cells, which has not been studied yet, our data showed that the MFI of CD26 + cells increased from day 0 to day 21 in the presence of SCF and FL, and also in the presence of IL2, IL7, and IL15 (Figure 3). For the SCF and FL groups, the MFI

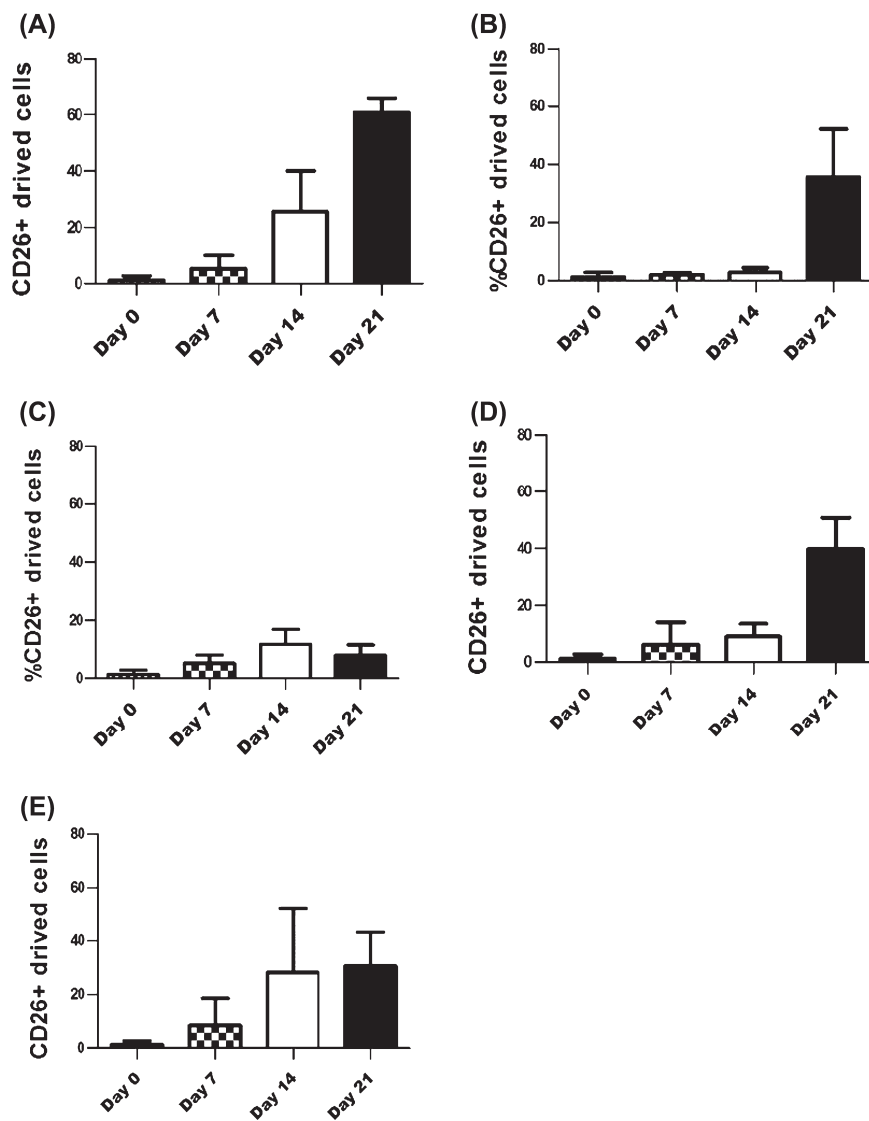


Figure 2. Cytokines can efficiently produce CD26 + cells. 10^5 cord blood mononuclear cells cultured with different combination of cytokines. Cultured cells harvested in identical time points and analyzed by FACS. Mean proportion of CD26 + cells have shown with combination of SCF + FL + IL2 + IL7 + IL15; $p < 0.0001$ (A), SCF + FL; $p < 0.0007$ (B), SCF + FL + IL2; no significant (C), SCF + FL + IL15; $p < 0.0001$ (D) and SCF + FL + IL7; $p < 0.01$ (E).

was in the same level. The same results were obtained when IL15 was added and also when three cytokines together (IL2, IL15, IL7) were added. However, on day 21, MFI increased with the use of IL2 and IL7 (43%), which was lower when using IL15 (53%).

Role of the cytokines in the expansion of CD26 + and CD26 – fractions of CD34 + cord blood mononuclear cells

Previous studies have reported that a subset of CD34 + cord blood cells express CD26. Moreover, inhibition of CD26 + cells improves engraftment of CD34 + cells (Christopher et al. 2004, 2007, Campbell et al. 2007). Since it is important to know the role of key immune cytokines on the CD26 + versus the CD26 – fraction of CD34 + mononuclear cord blood cells, we cultured mononuclear cord blood cells in the presence of SCF and FL, by adding IL2, IL7, and IL15 separately and also simultaneously. The flow cytometric analysis of cells harvested on days 0, 7, 14, and 21 (Figure 4)

showed that CD26 + and CD26 – fractions expanded on day 7 without the addition of any cytokine (35%, 21% respectively); however, both declined until day 21 (17%, 11%) (Figure 5A). In the presence of all cytokines in culture (SCF, FL, IL2, IL7 and IL15), the expansion of CD26 + and CD26 – did not show any difference, but both declined on day 21 (Figure 5B). The CD26 + fraction expanded from day 7 (13%) to day 21 (37%) with SCF and FL, whereas the CD26 – population declined from 19% to 7.6% (Figure 5C). CD26 + and CD26 – cells increased by the same amount on day 7, with the addition of IL2 (20%), but CD26 + cells slightly declined and CD26 cells sharply reduced to 8.6% till day 21 (Figure 5D). The CD34 + CD26+ fraction increased with SCF, FL, and IL7 on day 14, and reduced on day 21 (from 24% to 4%), although CD34 + and CD26 – population had not significantly changed during the study periods (Figure 5E). The CD26 + and CD26 – fractions of CD34 + expansion were in the same amount at indicated time points (day 7, 14),

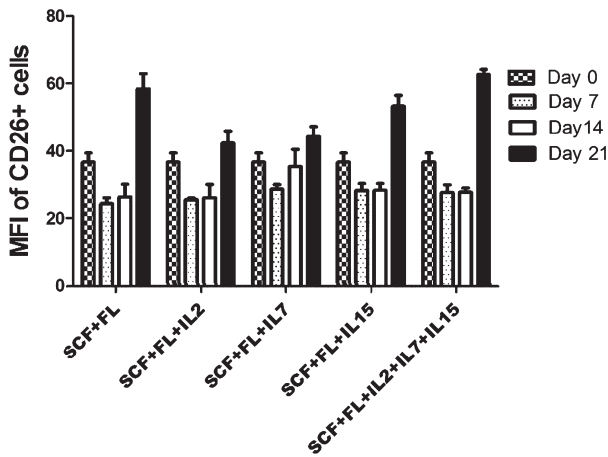


Figure 3. Mean fluorescence intensity (MFI) of different cytokines are in same level. Mean fluorescence intensity of CD26 + cell evaluated by FACS in indicated time points from CD26 + cell-derived cord blood mononuclear cells; $p < 0.0001$.

while CD26 – reduced on day 21 (Figure 5F). These data suggest the significant role of cytokines on the expansion of CD26 fraction of CD34 + cells.

Discussion

The present study demonstrated the role of important cytokines (IL2, IL7, and IL15) on the expansion of CD26 cells as a subpopulation of CD34 + cells in mononuclear cord blood cells.

Previous studies have shown that CD26 cells have a negative effect on the engraftment of CD34 + cells, either in bone marrow or in cord blood (Christopherson et al. 2007, 2012, Campbell et al. 2007, Prabhash et al. 2010). The amount of

cord blood cells are rare; therefore, to overcome this problem, and to achieve efficient transplantation, more cells from several full-term babies delivered should be collected, or the cells should be expanded by using a combination of cytokines (Xu and Reems 2001, Hofmeister et al. 2007, Mayani et al. 1993, Kita et al. 2011, Li et al. 2010, Oran and Shpall 2012, Ren and Jiang 2013). The results showed a significant effect of IL7 and IL2 on the CD26 fraction, and this effect was different from that seen with IL15.

Several studies have reported that various cytokines, including IL2, IL7, and IL15 have important roles in immune repopulating cells (Zhang et al. 2011, Sirskyj et al. 2008, Notarangelo et al. 2000). For T cell expansion and in the generation of NK cells, IL2 has crucial role. Moreover, IL2 is mostly being used as an elementary cytokine during transplantation for blood cancers (Cavazzana-Calvo et al. 1996, Meazza et al. 2011, Zhang et al. 2011, Le-Barillec et al. 2005, Metcalfe et al. 2012). Our data showed that IL2 increased the CD26 + fraction in the initial period of the study and then reduced it after day 14, but the CD34 + CD26 – population slightly increased; this is a valuable finding to improve the engraftment and repopulation of recipients.

IL7 is involved in proliferation and survival of T cells and also in B cell development, as well as in IL7-dependent NK cell population (Parrish et al. 2009, Cavazzana-Calvo et al. 1996, Sitnicka et al. 2003, 2007, Le-Barillec et al. 2005). Here, we found that IL7 has a significant role in expansion of CD26 + cells in cord blood mononuclear cells *in vitro*. It affected CD26 + expansion by at least 2-fold more than IL2 and IL15. However, the CD26 + cell expansion reduced when it was used in combination with IL2 and IL15. This reduction can be addressed in sharing IL7 receptors with IL2 and IL15. These receptors are occupied by IL2 and

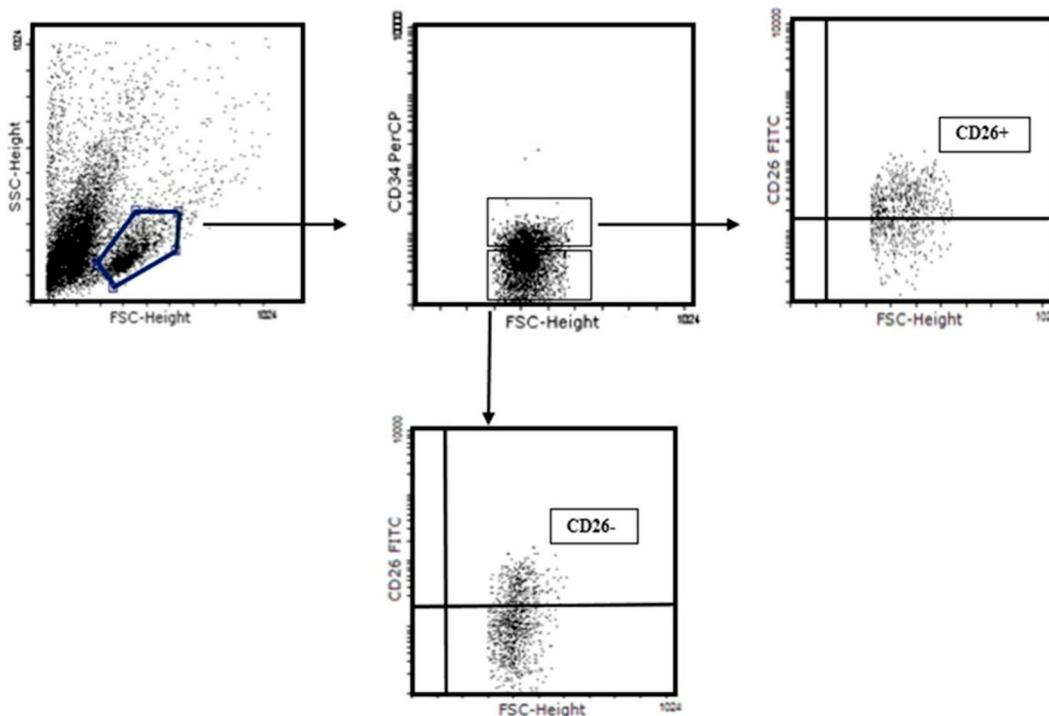


Figure 4. Representative FACS profile of 10^5 cultured cord blood mononuclear cells in identical time points. CD34⁺ CD26⁺ and CD34⁺CD26⁻ evaluated by gating on lymphoid population in FSC versus SSC.

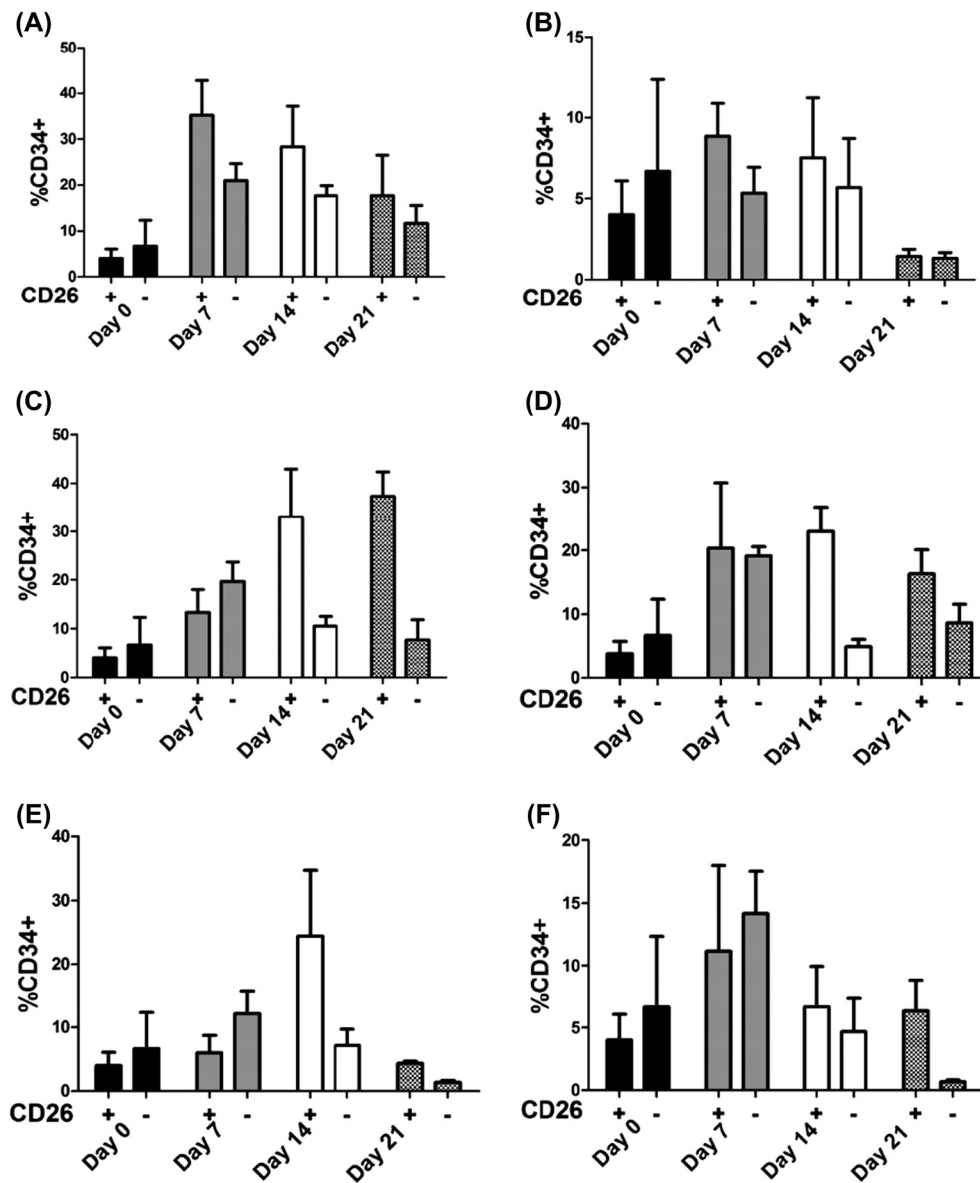


Figure 5. Evaluation of CD34⁺ CD26⁺ and CD34⁺ CD26⁻ cells derived from cord blood mononuclear cells at different time points. The harvested cells evaluated by FACS and mean (SD) have shown using no cytokines (A), SCF+ FL+ IL2+ IL7+ IL15 (B), SCF+ FL (C), SCF+ FL+ IL2 (D), SCF+ FL+ IL7 (E) and SCF+ FL+ IL15 (F).

IL15 with high affinity, whereas IL7 is a critical modulator of low-affinity peptide-induced proliferation (Fry and Mackall 2002).

IL15 is a key cytokine for NK cell development and has a crucial role in the proliferation and survival of different NK cell subsets (Cheng et al. 2009, Nozad Charoudeh et al. 2010, Vosshehrich et al. 2005, Ranson et al. 2003, Ranson et al. 2003). NK cells also play an important role in transplantation. Therefore, it is crucial to use IL15 in the expansion and induction of NK cells in cord blood cells. Our data illustrated that IL15 increases the rate of CD26⁺ cells in cord blood mononuclear cells. IL15 affects both fractions (CD26⁺ and CD26⁻) of CD34⁺ cells. CD26⁺ and CD26⁻ cells expanded over time. The cord blood expansion should be carefully performed utilizing IL15, if CD26⁺ cells are a crucial player. Overall, our findings suggested more CD26 proliferation using IL7 and IL15 separately; however, the influence of IL2 on CD26 reduced with time.

Conclusion

Taken together, our data suggest that using IL7 alone causes more CD26 cell proliferation; however, IL2 influence on CD26 was reduced with time, and it could improve the negative effect of CD26⁺ cells after transplantation.

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Authorship

Contribution: A.Z. and H.N.C. designed and conceptualized the research, analyzed the data, and wrote the manuscript.

A.Z. purified cell populations, carried out *in vitro* cultures, and analyzed the data; K.N, B.B, M.S, and H.T.N participated in the study design, and coordinated the sample discussions of data.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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