Rapamycin Inhibits Expansion of Cord Blood Derived NK and T Cell

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ABSTRACT

Background: The mammalian target of rapamycin (mTOR) is important in hematopoiesis. Despite the central role of mTOR in regulating the differentiation of immune cells, the effect of mTOR function on cord blood mononuclear cells is yet to be defined. **Objectives**: To evaluate the effect of mTOR inhibition, using rapamycin on the proliferation and apoptosis of cord blood mononuclear cells, as well as on the B and T cell expansion. **Methods:** Cord blood mononuclear cells were cultured in the presence of IL-2, IL-7 and IL-15 cytokines and inhibited by rapamycin for 14 days. The harvested cells were evaluated at distinct time points by flow cytometry. **Results:** The mTOR expression decreased in the presence of rapamycin on day 14. Inhibition of mTOR reduced the proliferation of the cord blood mononuclear cells, yet did not influence apoptosis. Moreover, the number of T and NK cells was significantly reduced in the presence of rapamycin, while no change was observed in the B cell expansion. **Conclusion:** mTOR signaling plays a crucial part in cord blood derived NK and T cells expansion.

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Keywords: Cytokine, mTOR, NK and T Cells, Rapamycin

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INTRODUCTION

The mammalian target of rapamycin (mTOR), known as a serine/threonine kinase, supports the survival, differentiation and cell growth in hematopoiesis (1). Signals generated by mTOR affect cell cycle progress from G1 into S phase during immune cell proliferation. It has been reported that mTOR signaling pathways are involved in nutrient availability, signals created by cytokines and stress responses (2,3).
The stimulation of the B and T antigen receptors and cytokine receptors activates the mTOR signaling pathway (9). Inhibiting the T cell proliferation, rapamycin acts as an immune-suppressive agent, preventing allograft rejection (4,5). The immunostimulatory and immunoregulatory effects of rapamycin suggest that the mTOR pathway plays a major role in regulating the activation of adaptive immune cells, including T, B, and natural killer cells (4). It has been demonstrated that $CD4^+$ T lymphocytes are noticeably reduced in the presence of rapamycin (6). Moreover, the inhibition of mTOR by rapamycin clearly weakened B cell proliferation and plasma cell differentiation (7). Rapamycin inhibits the proliferation of NK cells and impairs their cytotoxicity through inhibiting NKG2A and NCR expression in human peripheral NK cells (8).
Rapamycin reduces neutrophil differentiation from human $CD34^+$ cord blood hematopoietic cells, but does not significantly affect the levels of apoptosis (9). Furthermore, rapamycin improves the engraftment of the transplantation of human cord blood $CD34^+$ cells following stimulation with proper cytokines (1). Rapamycin reduces the rate of growth, proliferation and survival of tumor cells (4) and induced apoptosis (10). Although it was reported that rapamycin had a significant effect on Hodgkin's lymphoma cell lines, its preventive affect increased in combination with doxorubicin (11). Lymphopiesis is regulated based on the level of proliferation, survival and differentiation. Obviously, cytokines like interleukin-2 (IL-2), IL-7 and IL-15 are the main players in this regulation (12,13). IL-2 is an important cytokine, activating B cells, T cell growth and NK cells (14). IL-15 is the key cytokine in the development of natural killer and CD8 T cell development (15,16,17) and IL-7 is involved in the expansion of B, T and thymic NK cells (14). Moreover, IL-2 and IL-4 cytokines stimulate mTOR activity, and reduce apoptosis in the immune cells (18,19).
Herein, we evaluated the effect of rapamycin on cord blood-derived B, T and NK cell expansion as well as on the proliferation and apoptosis of cord blood mononuclear cells.

MATERIALS AND METHODS

Cord blood mononuclear cell isolation. Cord blood mononuclear cells (MNCs) were isolated as previously described (20). The samples collected from full-term normal deliveries were diluted with phosphate-buffered saline (PBS-SIGMA) and centrifuged on Ficoll-paque (GE healthcare, 1.078 g/ml) at 850 gm for 25 minutes. Afterwards, plastic pipes were used to collect the mononuclear cells that were then washed twice and re-suspended in RPMI1640 (Gibco) and 10% FBS (Gibco) either for culture or freezing.
Culture procedure and cytokine condition. The 5×10^5 MNCs were cultured in 96-well plates in 200 µL of RPMI1640 (Gibco), 10% fetal bovine serum (FBS; Gibco) 1% penicillin/streptomycin (Gibco), and the final concentration of the following cytokines: Stem cell factor (SCF, 50 ng/ml), Fms-related tyrosine kinase 3 ligand (Flt3L, 50

ng/ml), IL-7 (80 ng/ml), IL-15 (80 ng/mL), and IL-2 (80 ng/mL) (All cytokines were bought from eBiosciences). The cells were kept at 37°C for 14 days, and half of the culture medium was replaced weekly (21). So as to inhibit the activity of mTOR, twenty ng/ml of rapamycin (Tocris Biosciences, Bristol, United Kingdom) was freshly added to the cells every 3 or 4 days (9). On days 7 and 14, the harvested cells were stained with antibodies and analyzed by FACS for mTOR expression, KI67, active caspase 3, T (CD3), B (CD19) and NK (CD56).

Monoclonal antibodies and flow cytometry. Monoclonal antibodies used in this study were mTOR (Novus Biologicals , A-8 Littleton,USA), KI67 (ebioscience, San Diego, USA), active caspase 3 (BD bioscience, San Diego, USA), CD3 (R&D, Canada, USA) for T cells, CD19 (BD Biosciences) for B and CD56 (BD Biosciences) for NK cells. Briefly, the cells were incubated in 50 µl of staining buffer with the appropriate amount of a monoclonal antibody specific for a cell surface antigen (CD3, CD19, or NKP46 for 20 min at 4°C). We performed intracellular staining on mTOR, KI67 and Caspase 3 using 250 ul Fixation/Permeabilization solutions per tube for 20 min at 4°C; the cells were further washed twice in 1× BD Perm/Wash™ buffer (BD Biosciences) (21).

Flow cytometry was performed on days 7 and 14 of the culture time period. Shortly, the cells were incubated with these antibodies in each group (for 20 min at 4ºC). The cells were analyzed by BD caliber (BD ebioscience) between 10000 to 30000 events and were then collected and analyzed by flow Jo software (Flowjo: 7.6.1.).

Statistical Analysis. All data statistically analyzed by one-way ANOVA and Tukey test. We used Prism software (GraphPad Software, Inc., San Diego, CA version; 6) for all analysis and graphs. All experiments were done in triplicate and values expressed means ± SD (P<0.05).

RESULTS

mTOR expression in cord blood mononuclear cells.

We evaluated the expression of mTOR in cultured cord blood mononuclear cells with $SCF^{+}FLt3^{+}IL\text{-}2^{+}IL\text{-}7^{+}IL\text{-}15$. mTOR expression increased to around 35% from day 7 to 14. It is important to understand the consequences of mTOR inhibition.

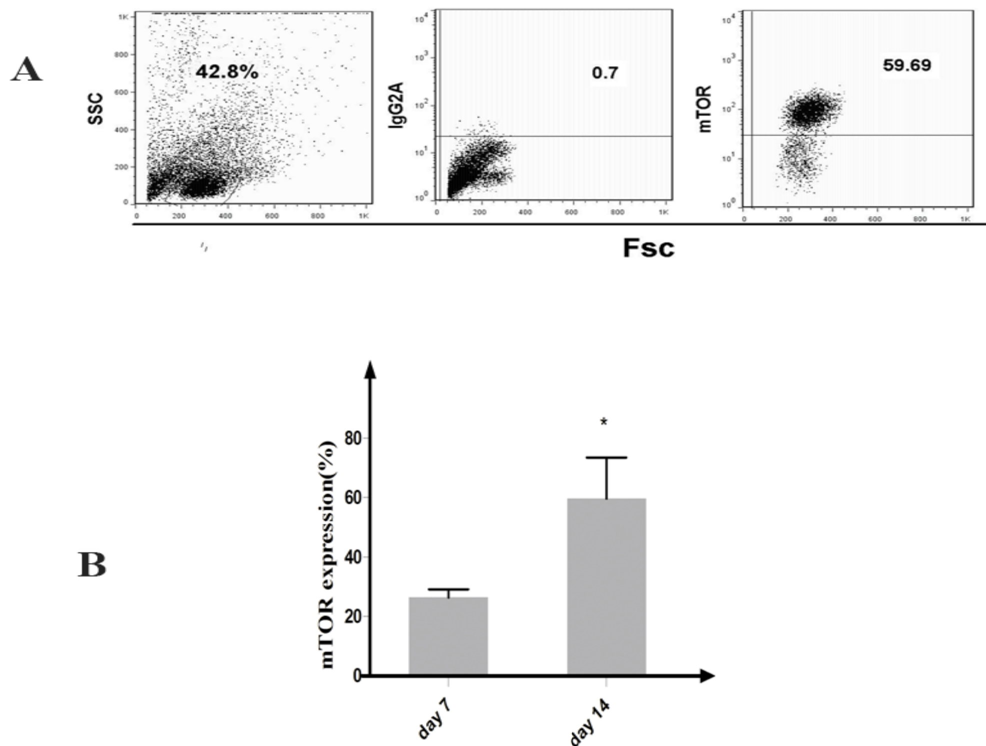


Figure 1. Rapamycin reduced the `mTOR expression in cord blood mononuclear cells. (A) Representative FACS plots for the evaluation of mTOR, **(B)** mTOR expression evaluated in harvested cord blood mononuclear cells. Culture was supplemented by SCF+FLt3+IL-2+IL-7+IL-15. Data represents the mean (SD) proportion of positive cells obtained from three independent experiments. The values were considered significant at **p<0.01.

Cord blood mononuclear cells proliferation dramatically reduced by rapamycin.
To understand the effect of the reduction of mTOR expression on the proliferation and apoptosis, we incubated the harvested cells with rapamycin. Flow cytometry analysis showed that KI67 expression was reduced from 63% to 7%; however, caspase 3 expression was not significantly changed (Figure 2), illustrating that rapamycin reduces the proliferation, but does not influence apoptosis.

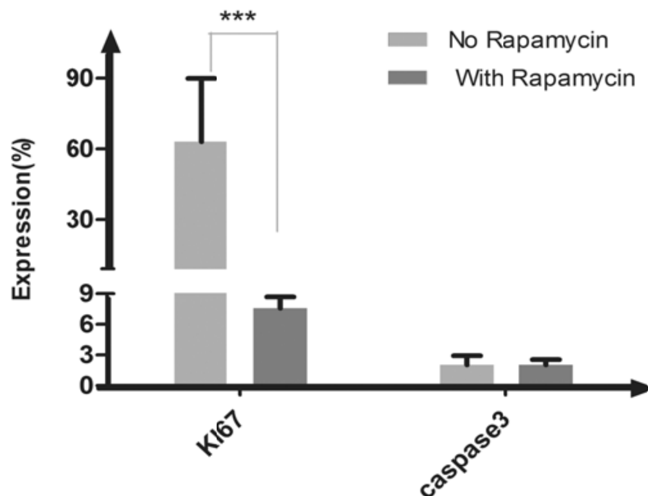
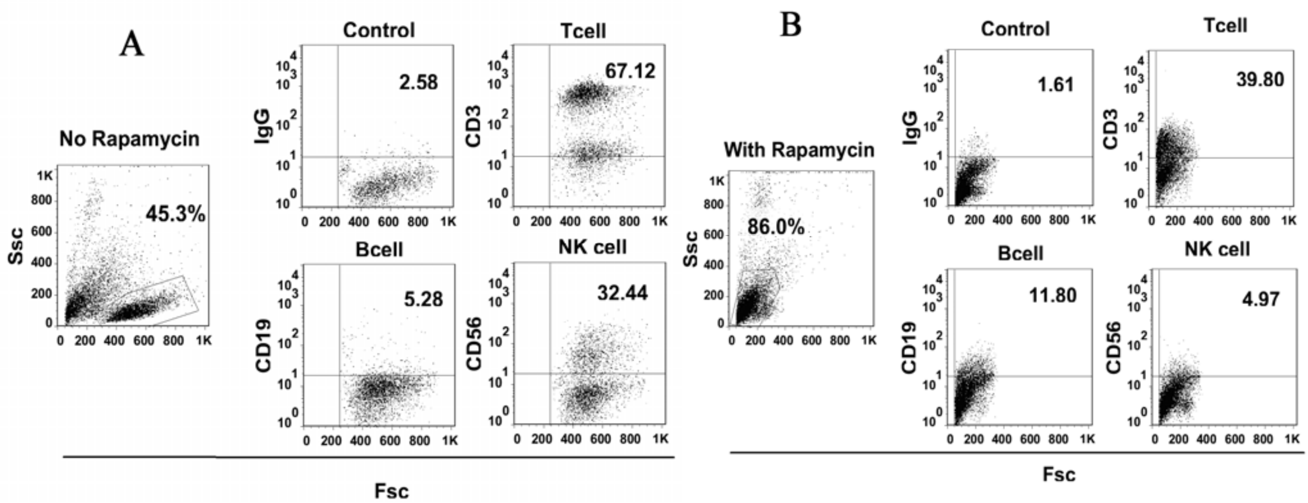


Figure 2. Rapamycin influences the Caspase 3 and Ki-67 expression of cord blood mononuclear cells on day 14. Cord blood mononuclear cells were cultured in the presence of Rapamycin and SCF, FLt3, IL-2, IL-7, and IL-15; harvested cells were evaluated for KI67 and caspase 3 expression by flow cytometry**.** Data represents the mean (SD) proportion of positive cells obtained from 3 independent experiments. The values were considered significant at ***p<0.001.

Cord blood derived B, T and NK cells influenced by rapamycin.
As mentioned before, B, T and NK cells are generated from cord blood mononuclear cells using SCF, FLt3, IL-2, IL-7, and IL-15 (14). Here, we examined whether rapamycin could alter B, T and NK cell expansion in cord blood cells. Harvested cells were evaluated for B ($CD19^+$), T ($CD3^+$) and NK ($CD56^+$) cells following the use of rapamycin by flow cytometry (Figures 3A & 3B). The $CD3^+$ cells were reduced from 67% to 40% in the presence of rapamycin (Figure 3C). The CD56 positive cells were dramatically decreased to approximately 27% (from 32% without rapamycin to 5% with rapamycin) (Figure 3C). However, the increase in $CD19^+$ cells was non-significant, about 2% (Figure 3C).



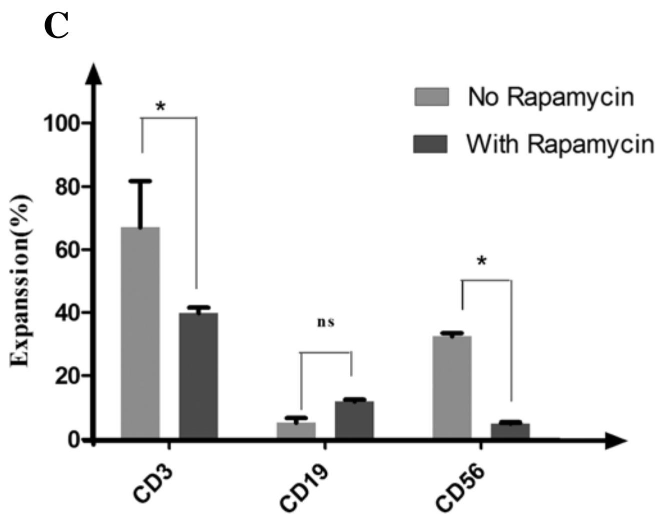


Figure 3. Cord blood derived T, B and NK cells were evaluated in the presence of Rapamycin on the 14th day 14 *in vitro*. (A,B) Representative FACS plots of T, B and NK cells derived from cultured 5×10^5 Cord blood mononuclear cells. **(C)** B, T and NK cells evaluated by rapamycin on day 14. Supplemented cytokines were SCF, FLt3, IL-2, IL-7, and IL-15. Data represents the mean (SD) proportion of positive cells obtained from three independent experiments. The values were considered significant at *p<0.05 and **p<0.01.

DISCUSSION

Rapamycin consists of a functional complex with intracellular 12-kDa FK506-binding protein (FKBP12), named rapamycin-FKBP12 complex which directly interacts and inhibits mTOR; on the other hand, the mechanism pertaining to how it inhibits mTOR activity is not exactly known (22,23). In this study, we demonstrated that rapamycin significantly decreased mTOR expression during the culture of cord blood MNC cells and dramatically reduced its proliferation. mTOR signaling, in turn, activates the synthesis of 4E binding protein (4E-BPI) as an inhibition factor and p70-kDa S6 ribosomal protein kinase (p70S6k) as an essential factor in regulating cellular growth and proliferation (10,24), thereby elucidating the anti-proliferative activity of rapamycin in cord blood MNC cells. In human endothelial progenitor cells, the epithelial cells, myoblasts and osteoblasts and rapamycin retard the cell cycle progression (25,26,27,28). Furthermore, the activity of IL-1, IL-2, IL-3, IL-4, IL-6 is suppressed by rapamycin (23). The results in this study revealed that in order to increase cord blood mononuclear cells during culturing with cytokines, it is necessary to employ rapamycin-sensitive mTOR signaling. Accordingly, the proliferation rate was significantly reduced in the presence of rapamycin.

It was also indicated that rapamycin differentially affects several populations and it was claimed that the expansion of T and NK, unlike B cell, progenitors decreases in the presence of rapamycin (Figure 3); mTOR signaling plays an critical role in sensing the microenvironment, activity and differentiation of immune cells (2,4). In addition, the mTOR signaling is a key regulator between effector and regulatory T cell lineage commitment (29). The mTOR-deficient $CD4^+$ T cells that are associated with lower activation of the transcription factors STAT6, STAT3 and STAT4 fail to differentiate the effector cells in an appropriate culture condition including IL-4, IL-6 and IL-12. On the other hand, they differentiate the $Foxp3^+$ regulatory cells after being activated by exogenous IL-2 (3,29). Rapamycin, via inhibiting the T-bet expression downstream of mTOR, manages to both inhibit effector functions (like IFN-γ) in T cells and increase the generation of memory T cells (30). Therefore, mTOR signaling appears to be essential for the differentiation of immune cell progenitors in cord blood. However, the downstream regulating signaling pathways involved in such activities require further investigation. In conclusion, the mTOR expression in cord blood is affected by IL-2, IL-7 and IL-15; furthermore rapamycin reduces the proliferation of cord blood mononuclear cells following mTOR reduction. mTOR is an important factor in the development of immune cells, particularly in cord blood-derived T and NK cell development. The number of T and NK cells reduced following mTOR inhibition by rapamycin.

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