Higher toll-like receptor 3 expression in umbilical cord blood B cells than in adult blood B cells

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Background
Umbilical cord blood (UCB), which is considered a rich source of stem cells, has been used for applications in different clinical settings. Therefore, it is crucial to examine the toll-like receptor (TLR) expression levels in UCB B cells as compared to adult blood cells.
Aim
To determine the phenotypes of B cells in UCB and to investigate their expression of TLR3 as compared to adult blood.
Patients and methods
Samples of UCB were collected (n=20) after delivery, and peripheral blood samples were collected from female healthy volunteers (n=10) in K2EDTA tubes. Cells were washed twice, then stained using anti-CD19 and anti-TLR3. The samples were acquired by flow cytometry to assess the phenotype of B cells and their expression of TLR3. Besides, the liver and kidney functions were assessed.
Results
The relative number of $CD19^+$ cells showed lower numbers (5.35%) in UCB than adult blood (15.64%). Additionally, the absolute number of $CD19^+$ cells showed lower numbers by two-fold in cord blood than in adult blood. The relative expression of TLR3 on $CD19^+$ cells showed lower expression in UCB as compared to adult blood by 5.8-fold. However, the absolute number of TLR3+$CD19^+$ was higher in UCB than in adult blood by two-fold. The liver and kidney function showed normal values as investigated the enzyme activity of aspartate aminotransferase, alanine aminotransferase, bilirubin, and creatinine in both cord and adult.
Conclusion
B cells express lower TLR3 in cord blood than in adult blood. The data from this study open new avenues for the manipulation of cord blood by TLR agonists for clinical application.

Keywords:
B cells, UCB, immune cells, $CD19^+$, adult blood, toll-like receptors

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Abbreviation: ALT, alanine aminotransferases; AST, aspartate aminotransferases; MSCs, mesenchymal stem cells; PBS, phosphate-buffered saline; PLT, platelets; TLR, toll-like receptor; UCB, umbilical cord blood; WBCs, white blood cells.

Introduction

Umbilical cord blood (UCB) has been established in the last 20 years as a rich source of stem cells for application in different clinical settings, such as autoimmune diseases, stem cell transplantation, and hematological malignancies. A newborn's immune system becomes established and is exposed to several environmental stimuli. Compared to newborns that are born via cesarean section, infants delivered after normal pregnancies showed increased levels of natural killer cells and granulocytes due to the physical stress and normal cortisol release during labor [1]. Despite the fact that most of the studies on the toll-like receptors (TLRs) have been done on adult immune cells [2], few studies addressed their expression on UCB, where most of them focused on mesenchymal stem cells (MSCs) [3,4]. Previous research has also indicated that MSCs obtained from bone marrow and adipose tissue exhibit increased osteogenic differentiation upon stimulation with TLR3 and TLR4 [5]. Additionally, it has been noted that TLR3 and TLR4 improve the immunosuppressive properties of human bone marrow-derived MSCs [6].

The TLRs are a class of pathogen-associated molecular patterns that can initiate immune responses. They can be activated by components of several pathogens as well

as by endogenous ligands [7]. In human cells, 11 TLR members bind different microbial products from bacteria, viruses, and fungus [8]. TLR3 recognizes double-stranded RNA in viruses, while TLR4 can bind to lipopolysaccharide, a component of Gram-negative bacteria [9].

Given that TLRs modulate the activation of immune cells and secretion of proinflammatory and preinflammatory cytokines such as interleukin-1, interleukin-6, tumor necrosis factor-α, interferon-γ for a better immune response [10], it is of paramount significance to analyze the expression levels of TLRs on the UCB relevant to adult blood cells. Therefore, the purpose of this study was to determine the numbers of B cells in UCB and to investigate their expression of TLR3 as compared to those in adult blood. We found that B cells express lower TLR3 expression in cord blood than in adult blood.

Patients and methods

In this study, there were 20 cord blood samples and 10 peripheral blood samples of healthy adult volunteers. Cord blood samples were obtained immediately after delivery in the Department of Obstetrics and Gynecology, Tanta University Hospital, Tanta University, Tanta, Egypt. The blood samples were taken from the umbilical cords of newborns whose parents provided informed consent, following a protocol approved by Tanta University Ethical Committee, the Faculty of Medicine, Tanta University, Egypt (Number 3012–01-15 36264PR729/6/24). The median age of the healthy individuals was 24.32±3.81 years. The selection of the volunteer participants was carried out following the identification of the inclusion and exclusion criteria, which included a clear history of treatment with board spectrum antibiotics for at least 1 month prior to the donation and a clear history of chronic diseases. According to sex, they were all females included in the study, so there were no significant changes between the cord blood samples and the normal adult group (Table 1).

Chemicals and reagents

Phosphate-buffered saline (PBS) was purchased from the Verviers, Belgium-based manufacturer Lonza. The clones (SK3) and HTA125 for TLR3 (phycoerythrin) and CD19 (perCP.Cy5.5) were purchased from BD Biosciences Company (BD Biosciences, San Jose, California, USA). The sheath fluid was purchased from BD Biosciences.

Collection of blood samples

After delivery, UCB were collected and placed on K2EDTA tubes for the separation of peripheral blood leukocytes (PBL). The PBL were separated using the ammonium chloride potassium solution for red blood cell (RBC) lysis, incubated for 15 min, and were then twice washed with cold PBS before being stored in 500 µl of PBS in a refrigerated environment.

Complete blood count

The total white blood cells (WBCs) were counted in the peripheral blood using an automated instrument for complete blood count (ABX Micros 60 hematology analyzer; Horiba Medical, USA). The absolute cell number of each population was calculated as [total WBCs count (cells/ml)×percentage of cells]/100 [11].

Measuring of biochemical parameters

Biochemical parameters, including measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity, determination of creatinine level, and determination of total bilirubin level were done for both cord blood and adult blood by spectrophotometer.

Surface staining by flow cytometry

Peripheral blood samples from each time point were collected for $CD19^+$ immunophenotyping by flow cytometry. Cells were lysed with FACS lysing solution, which was purchased from (BD Biosciences) and then washed with PBS)FIPCO, El Fath for Pharmaceutical & Cosmetics Industries, Alexandria, Egypt). Cells were incubated with human FcR blocking reagent purchased from (Miltenyi Biotec, Bergisch Gladbach, Germany) before surface staining. Cells were then stained with anti-CD19 and anti-TLR3 (BD Bioscience) using the concentrations recommended

Table 1 Demographic and clinical data of the studied populations

	Cord blood (*N*=20) [*n* (%)]	Control (*N*=10) [*n* (%)]	*P*
Sex			
Female	20 (100)	10 (100)	≥0.05
Age			
Minimum–maximum	0 day	21.0–27.0	<0.05
Mean±SD	–	24.32±1.43*	

P, *P* value for comparing between the two studied groups. *Statistically significant at *P* value less than or equal to 0.05.

Table 2 The hematological incidence in umbilical cord blood groups compared to the control group

	Healthy control	UCB patients
Hb (g/dl)		
Minimum–maximum	10.3–15.5	8.4–12.4
Mean±SD	11.725±2.43	10.9±1.5
$P_{Control}$		0.643
WBCs/mm^3		
Minimum–maximum	4750.0–10750.0	2020.0–3700.0
Mean±SD	7011±2245	7565±2311
$P_{Control}$		0.401
PLTs/mm^3		
Minimum–maximum	150 000–420 000	35 400.0–38 000.0
Mean±SD	270.083±87390	211.625±10.97.69
$P_{Control}$		0.012*

PLT, platelet; UCB, umbilical cord blood; WBC, white blood cell. $P_{Control}$: *P* value for comparing between control and each other group. *Statistically significant at *P* value less than or equal to 0.05.

by the manufacturers of each antibody. The stained samples were incubated in the dark for 20–30 min. Then, BD FACS lysing solution was added for 15 min for RBC lysis, then centrifuged at 1500 rpm for 5 min, and the supernatant was discarded to remove the lysed RBCs. Cells were washed twice using PBS and then were suspended in PBS. Unstained samples were used as internal controls throughout the experiments. Data were acquired on a FACSCanto cytometer (BD Bioscience, Franklin Lakes, New Jersey, USA). The obtained data were analyzed using BD FACSCanto software. The absolute numbers of $CD19^+$ in the blood were calculated as % $CD19^+$×total WBCs/100 [11].

Statistical analysis

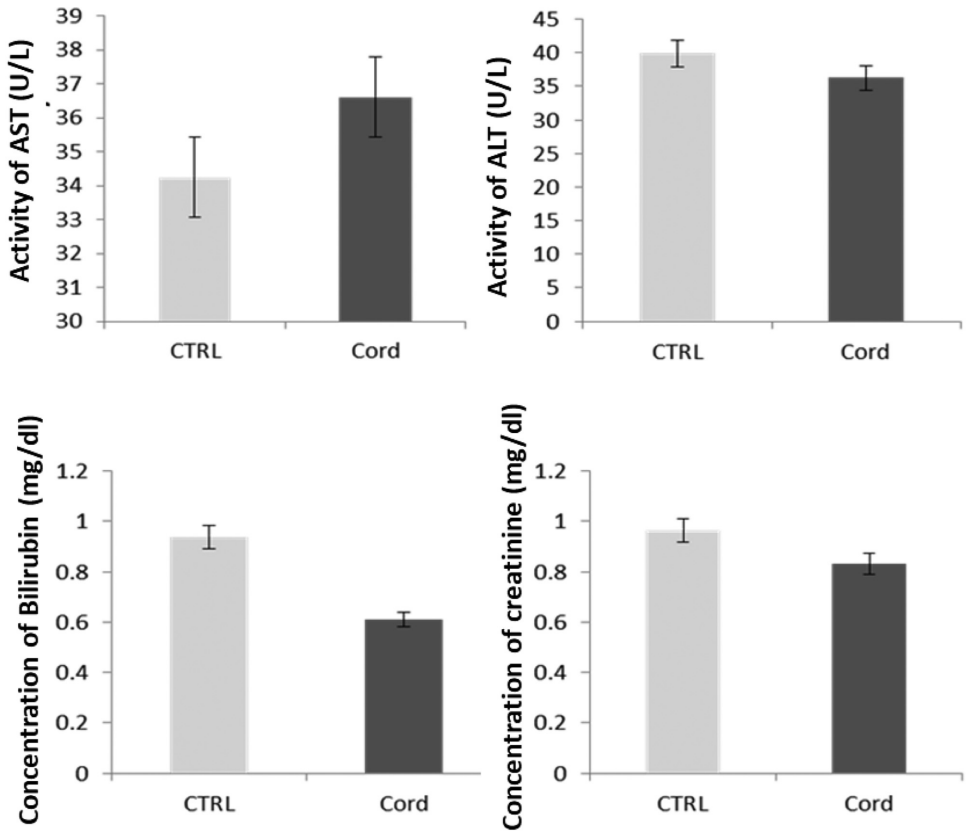
The data were collected during the study and analyzed for each volunteer; each value was calculated as the mean±SD. The results were analyzed using a repeated measures *t* test by using graph pad prism software. The *P* values less than 0.05 were significant.

Results

Similar values of hemoglobin, platelet, and white blood cells and liver function in the cord and adult blood

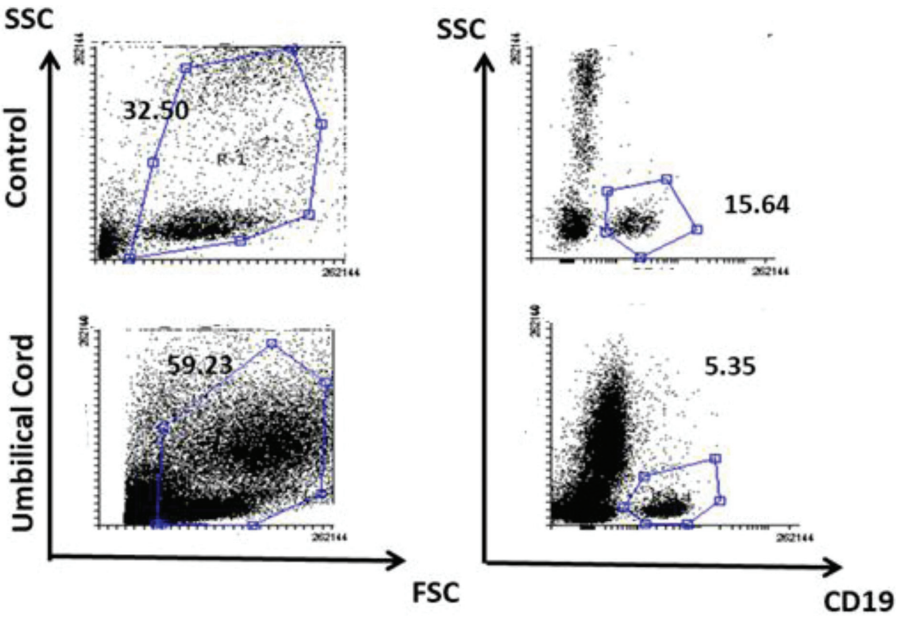
To confirm that participants were healthy, we measured certain clinical parameters. We found that there were no significant differences in hemoglobin, platelet (PLT), and WBC levels between cord blood and adult individuals (Table 2). By analyzing the serum enzymes in the cord and adult blood that indicate the liver (ALT and AST) and kidney (creatinine) function,

Figure 1



The liver and the kidney functions, including AST, ALT, creatinine, and bilirubin in the UCB patients as compared to the healthy control donor's blood. ALT, alanine aminotransferase; AST, aspartate aminotransferase; UCB, umbilical cord blood.

Figure 2



Representative data illustrating the gating strategy of $CD19^+$ in the blood of control and cord using flow cytometry. Gates are from total populations that showed $CD19^+$.

we found no difference between their values in cord blood and adult blood (Fig. 1).

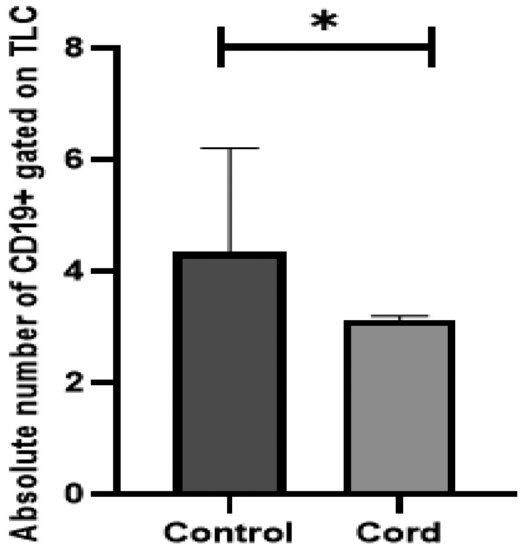
Cord blood shows lower numbers of $CD19^+$ cells than adult blood

The flow cytometry analysis of $CD19^+$ numbers was determined for UCB and normal adult PBL. We found that the relative number of $CD19^+$ cells was lower in cord blood (5.35%) than in adult blood (15.64%) (Fig. 2). Additionally, the absolute number of $CD19^+$ cells was significantly two-fold lower in the UCB than in adult blood (Fig. 3).

Cord blood shows higher expression of TLR3 on $CD19^+$ cells than in adult blood

The relative expression of TLR3 was significantly lower in cord blood (6.8%) than in adult blood (35.64%), as shown in Fig. 4. However, the absolute expression of TLR3 on $CD19^+$ was two-fold higher in the cord blood than in the adult blood (Fig. 5).

Figure 3

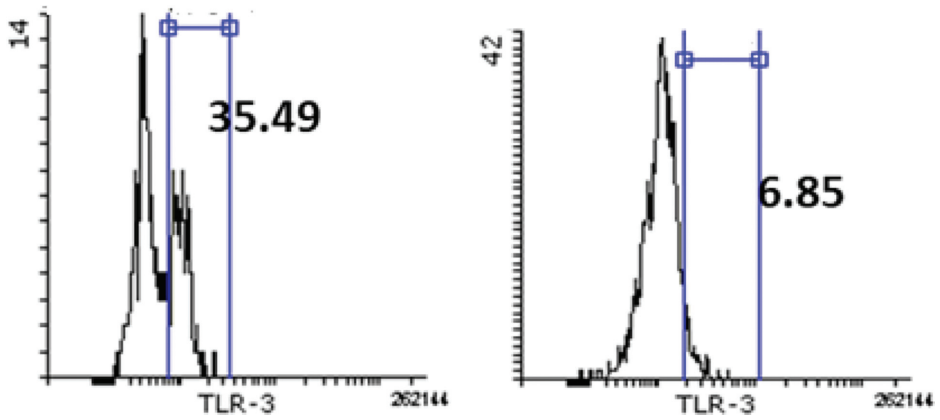


Absolute number of $CD19^+$ B cells in the UCB as compared to normal adult blood. (*$P \leq 0.05$). UCB, umbilical cord blood.

Discussion

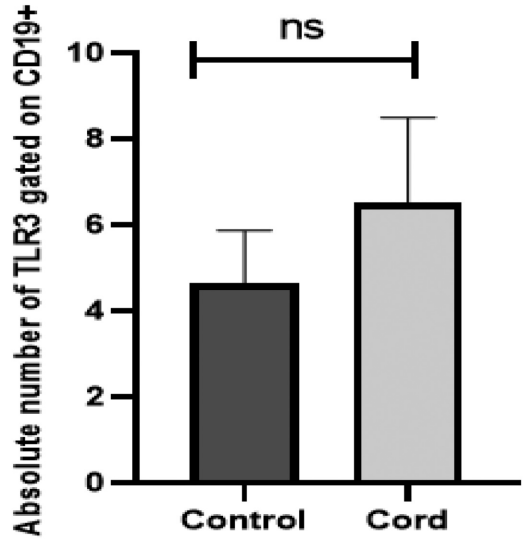
UCB, which is considered a rich source of stem cells, has been used for applications in different clinical settings [12]. Although several studies have investigated the expression of TLRs on MSCs and how TLR agonists modulate these cells [13], few studies addressed the TLR expression on different UCB subsets. As such, this study aimed to determine the expression of TLR3 on B cells in HUCB and compare it to B cells in the adult blood using flow cytometry. We found that although cord blood contains lower numbers of B cells than adult blood, they express higher levels of TLR3 than B cells in the adult blood. Given the important role of B cells in the generation of effective immunity and the immunomodulatory role of TLR3 as a sensor for dsRNA, our data open a new avenue for the

Figure 4



Representative numbers of TLR3 expressed on CD19$^+$ B cells in the UCB as compared to normal adult blood. TLR3, toll-like receptor 3; UCB, umbilical cord blood.

Figure 5



The absolute number of TLR3+CD19$^+$ B cells in the UCB as compared to normal adult blood ($P \leq 0.05$). TLR3, toll-like receptor 3; UCB, umbilical cord blood.

manipulation of cord blood B cells by TLR3 agonists for relevant clinical applications.

We first investigated some of the hematological indices as well as the liver and kidney functions in the sera from the cord and adult blood samples as a conventional measure for overall performance. We found normal values of hemoglobin levels, AST and ALT, PLT, and WBCs in the sera of cord blood and volunteer samples. Our data is consistent with previous studies reporting that there were no differences in hemoglobin, PLT count, and WBCs count in cord blood [14–16]. These data indicate that the mother might have more immune cells to strengthen the child's immune system and help her infants to fight infections. These findings are consistent with another study, which found that the increased WBC count in newly born infants is normal and related to the mother's medical history of infection and her current state of pregnancy [17]. These clinical settings were important to predict that there was no infection before or during delivery.

Our study showed that both the relative and absolute numbers of B cells were lower in cord blood than adult blood. Our results were in line with previous studies, which showed that B cells were lower in cord blood, which was associated with a higher number of natural killer cells and a lower number of T cells. [18,19]. Taken together these studies with our study, it seems that the low number of B cells is a phenomena for cord blood, which could be due to the higher number of other cell subsets. Although we have not analyzed the B cell subsets (naïve vs. mature), [19] analyzed the frequencies of circulating B cell subpopulation in the cord blood, where he found increasing frequencies of IgM memory B cells, class-switched memory B cells, marginal zone B cells and plasmablasts in cord blood to peripheral blood of juveniles and adults. In contrast, the percentage of naïve B cells was higher in newborns than in juveniles and adults [20].

To the best of our knowledge, few studies have examined TLR expression in B lymphocytes. By analyzing the TLR3 expression on B cells, we found that the relative number of TLR3+ B cells was lower in cord blood than in adult blood. However, the absolute number of TLR3+ on B cells was higher in cord blood than in adult blood. TLR3 has been found to be expressed by B cells, which become highly activated upon TLR ligation, resulting in improvements in B cell

survival, cytokine production, presentation of antigen, and binding of TLR with TLR agonists [21–23]. According to these studies, B cells may receive signals through TLRs during an immune response, providing a link between the innate and adaptive immune responses. Consistent with these studies, we have found recently that B cells can respond to the TLR3 ligand Poly(I : C), resulting in their antigen-independent and antigen-dependent proliferation and activation *in vitro* and T cell expansion *in vivo* (data not shown) [6].

Besides the fact that B cells receive signals through B cell antigen receptor and they can also receive TLR signaling, we suggest that the best costimulatory approach for the optimal activation of B cells could be through B cell antigen receptor ligation, TLR ligation, and costimulation of CD40. This critical role of TLR in the stimulation of B cells is consistent with our recent data that reporting B cells from splenocytes can respond to CpG (TLR4), showing a higher response of proliferation and activation as well as enhanced responses of antigen-specific CD8+ T cells [6]. In contrast to our data, recent studies by Zhang *et al.* [24] indicated a lower expression of TLR3 on human naïve B cells in adult blood, which was associated with lower TLR-based B cell activation. This data supports our data, which shows that the expression of TLR3 is upregulated on B cells in UCB cells in response to their initial role in the innate and adaptive immune response.

Given the expression of TLR3 on B cells and the biological significance of TLRs in the adaptive immune response, our data opens a new avenue for better targeting of B cells in UCB for clinical applications for the treatment of different diseases in the future.

Conclusion

B cells express lower TLR3 in cord blood than in adult blood. The data from this study will open new avenues for the manipulation of cord blood by TLR agonists for stem cell-based transplantation.

Acknowledgment

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Authors' contributions

Sohaila M. Khalil: writing original draft and formal analysis and methodology. Menna Askar: helping in the experiment. Shimaa M. Abduljalil: revision and reviewing. Randa Ezz Eldien El Naggar: supervision and reviewing. Mohamed L. Salem: responsible for the idea concept and the experimental design.

Data availability

All data generated or analyzed during this study are included in this published article. More detailed data is available from the corresponding author upon reasonable request.

Conflicts of interest

There are no conflicts of interest.

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