Article

Sex differences in frequencies of B and T cell sub-populations of human cord blood

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Abstract: *Background:* Cord blood represents a link between intrauterine and early extrauterine development. Cord blood cells map an important time frame in human immune imprinting processes. It is unknown whether sex of the newborn affects the lymphocyte subpopulations in cord blood. *Methods:* 9 B and 21 T cell subpopulations were characterized by flow cytometry in human cord blood from 16 male and 21 female newborns, respectively. *Results:* Except for marginal zone B cells and transitional B cells, B cell count in all subsets was higher in cord blood of male newborns than in female newborns. Frequency of naive thymus negative Th cells was significantly higher in male cord blood whereas the remaining T cell subpopulations showed a higher count in cord blood of female newborns. *Conclusion:* Our study is the first revealing sex differences in B and T cell subpopulations of human cord blood. These results indicate that sex might have a higher impact for the developing immune system urging the need to expand research in this area.

Keywords: B cells; T cells; B1 cells; human; white blood cells; cord blood; development; ontogeny; sex differences; gender medicine

1. Introduction

The development of the human immune system begins intrauterine and is not completed at birth.[1] After birth, many processes are initiated, also affecting immune cells in particular due to changed environmental antigen exposure.[2] In the neonatal period, the immune system is immature and certain components of the adaptive immune response are not fully developed yet. Moreover, there are notable differences in the antibody response compared to adults. Although neonates benefit from maternal antibodies providing maternal passive immunity, antibody titers are lower than in adult blood.[3,4] During infancy, the immune system matures. As a continuous learning process, immunity is built up during puberty, also by intercurrent infections and vaccinations. The immune system achieves the similar functional capacities compared to adult mature immune system. [5]

The maturation of immune cells is a complex multi-layered process. Griffin et al. [6] described human B1 cells as CD20+CD27+CD43+ B cell subpopulation based on typical B1 functions in murine B1 cells: spontaneous IgM secretion, efficient T cell stimulation and continuous intracellular signal[6]. B1 cells have a huge percentage in cord blood (CB) (50%) but decrease over the years.[6],[7]

Marginal zone B (MZ B cells) cells are noncirculating mature B cells, forming an interface between blood and lymph nodes.[8],[2] Splenic MZ B cells located in the marginal zone of the spleen are part of the innate immune system responding to blood-borne antigens. Although accounting only for 5% of the innate and adaptive immune system, marginal zone B cells are of utmost importance

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for early immune response by interacting effectively with rapid antibody reactions on viral and bacterial pathogens.[8]

Thymus negative cells are a premature stage of T helper (T_h-) lymphocytes before positive selection in thymus. In the positive selection process, T_h cells can bind major histocompatibility complexes (MHC) in order to receive positive growing signals and enter cell cycle. After positive selection process, cells that bind self-antigens are induced to apoptosis.[9] These selection processes take place in the thymus postnatally assuming that maturation of T_h cells does not finish by the time of birth.[10]

In this context, viral and microbial colonization by perinatal transmission are important steps in the maturation process of the innate and adaptive immune system. Microbial dysbiosis within the first 100 days of life seems to play an important role, for the susceptibility to develop allergies later in life.[11]

One important and long neglected fact are differences between females and males. Investigating biological (sex) and sociocultural (gender) differences has gained more importance over the past few years. Within the field of medical research, most of the performed researches and published data refers to male individuals. Sex differences have been found in certain diseases like asthma, diabetes and cardiovascular disease. Women are at higher risk of developing diseases like Takotsubo cardiomyopathy or show different manifestations of common diseases like heart failure or myocardial infarction. More over autoimmune diseases as systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis and Hashimoto´s thyroiditis are more common in women than in men.[12],[13]

There is a constant increasing testosterone serum level in male newborns over the first three months after birth, with a decrease at 7-12 months, followed by maintaining pre – puberty low levels. In female newborns, there is a decrease of testosterone serum levels after birth. These gender differences are seen within the first year of life, as there is no difference in testosterone levels pre-puberty between male and female until onset of puberty.[14] Overall, Oestrogen stimulates the proliferation of B cells, while testosterone supresses.[15] Regarding T cell subpopulations, testosterone also supresses lymphopoiesis, in early stages. Passing several developmental steps, T cells abandon to express androgen receptors.

Another key role beside sexual hormones plays the X chromosome. On X chromosome, there are coding regions for genes involved in immune responses, as toll-like receptors, cytokine receptors and transcription factors.[17] During embryogenesis, there is a random silencing of one X chromosome, and at the same time, there is an escape out of this silencing process by 15 % of the respective immune related genes, with 10 % of the genes underlaying different levels of inactivation.[18] Thus, an overexpression of immune related genes is associated to X disomy. Genes associated to autoimmunity are also found on X chromosomes, as expression of TLR-7 described in lupus-associated autoimmunity.[19] Although female neutrophils and macrophages showed a better phagocyte activity, [20] testosterone reduces the production of TNF, expression of TLR4 and increases cytokines. Therefore, autoimmune mediated diseases are more frequent in women.[14]

A recent example for sex disparity and associated different clinical courses and outcomes are infections with severe acute respiratory syndrome coronavirus 2. Underlying mechanism remain elusive, the influence of oestrogens and androgens, as well as androgen-sensitive genes coding for angiotensin-converting enzyme 2 (ACE2) and cell surface transmembrane protease serine 2 (TMPRSS2) are under discussion. Besides, sociocultural influences are probable.[21]

There is only limited evidence for sex differences in leucocytes in cord blood. Hence, our study aims to gain more information about the composition of B and T cell subpopulations in cord blood depending on sex.

2. Results

2.1. B cells

We analysed cord blood samples of 16 male newborns and 21 female newborns by flow cytometry. The proportion of B1 cells was significantly higher in cord blood of male neonates than in cord

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blood of female neonates (male 3.7%±2.9 vs. female 1.82%±1.5, p=0,023, (figure 1). As shown in table 5 B1 cells are marked CD20+CD27+CD43+ and are given as a percentage of the number of total CD20+ cells. Marginal zone memory B cells, marked as CD19+CD27+IgD+IgM+ and given as percentage of total CD19+ cells, were also significantly higher in male cord blood (male 1.67%± 2,1 vs. female 0.79%± 1,2, p=0.01, figure 2). Except for the naive B cells and transitional B cells, B cell count in all other subsets (innate B cells, class switched memory B cells, late memory B cells, plasmablasts, transitional B cells, B2 cells) was higher in cord blood of male newborns (table 1).

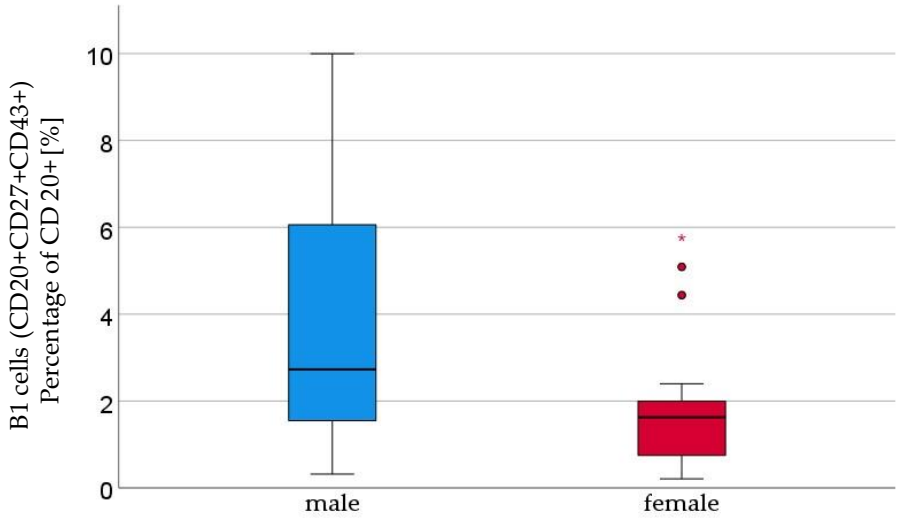

Figure 1. B1 lymphocytes (CD20+CD27+CD43+) percentage of CD 20+ [%] were significantly higher in male (n=16) than in female (n=21) newborns, given with median (male = 2.7, female = 1.6), minimum (male = 0.3, female = 0.2) and maximum (male = 10, female = = 5.8); mean ± standard deviation [%] (male = 3.7 ± 2.9, female = 0.8 ± 1.2).

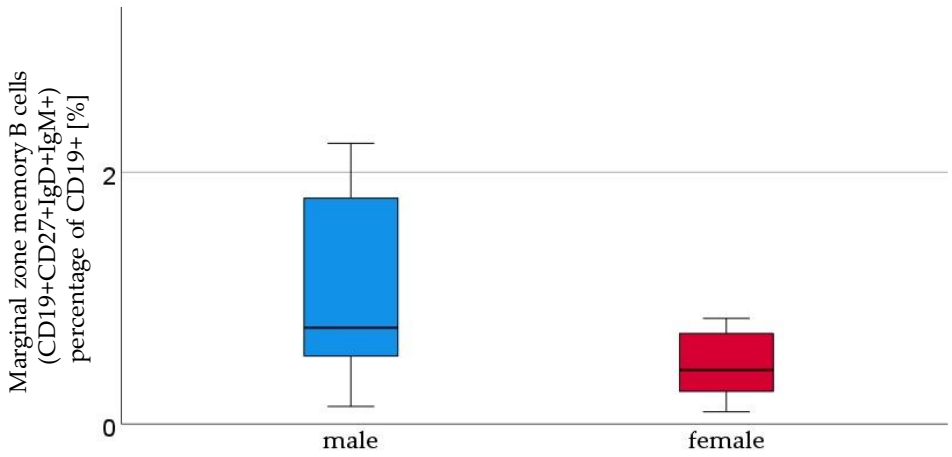

Figure 2. Marginal zone B lymphocytes (CD19+CD27+IgD+IgM+), percentage of CD 19+ [%], were higher in male (n=16) than in female (n=21) newborns, given with median (male = 0.8, female = 0.4), minimum (male = 0.1, female = 0.1) and maximum (male = 7, female = 4.3); mean ± standard deviation [%] (male = 1.7 ± 2.1, female = 0.8 ± 1.2).

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2.2. T cells

We analysed T cell subpopulations in two panels (table 5). In T cell panel 1 we analysed 16 male and 21 female and in T cell panel 2 we analysed 11 male and 16 female newborns, by flow cytometry.

Table 1. Relative amounts of T lymphocyte subpopulations with mean and standard deviation [%]. Group differences were tested using Mann-Whitney U test. Test groups were divided in male (n=16) and female (n=21) newborns.

	sex	Mean ± standard deviation [%]	p-value
Innate B cells (percent of CD19+ cells)	male	6.6 ± 4.0	0.8
	female	6.2 ± 3.7	
Naive B cells (percent of CD19+ cells)	male	72.0 ± 11.7	0.53
	female	75.3 ± 8.3	
Marginal zone memory B cells (percent of CD19+ cells)	male	1.7 ± 2.1	0.01
	female	0.8 ± 1.2	
Class switched memory B cells (percent of CD19 + cells)	male	5.7 ± 4.1	0.84
	female	5.3 ± 2.6	
Late memory B cells (percent of CD19+ cells)	male	3.2 ± 2.7	0.073
	female	1.7 ± 1.4	
Plasmablasts (percent of CD19+ cells)	male	5.5 ± 4.0	0.84
	female	5.2 ± 2.6	
Transitonal B cells (percent of CD19+ cells)	male	82.7 ± 11.0	0.89
	female	85.4 ± 6.7	
B1 cells (percent of CD20+ cells)	male	3.7 ± 2.9	0.023
	female	1.8 ± 1.5	
B2 cells (percent of CD20+ cells)	male	0.3 ± 0.3	0.13
	female	0.2 ± 0.3	

Cytotoxic T cells, naive effector T_h cells, naive effector cytotoxic T cells and naive thymus negative T_h cells showed a trend to be higher in cord blood of male newborns. Naive thymus negative T_h cells were significantly more abundant in male cord blood (male 24.4% ± 9.8 vs. female 15.9± 5.7, p=0.05, figure 3). 12 other T cell subpopulations (T helper cells, T helper cells with $\alpha\beta$-TCR, activated cytotoxic T helper cells with $\alpha\beta$-TCR, memory effector T helper cells, naive central T helper cells, naive central T helper cells, nemory effector cytotoxic T cells, memory central cytotoxic T cells, naive central cytotoxic T cells and naive thymus positive T helper cells) showed a non-significant higher count in cord blood of female newborns than in male newborns (table 2). The remaining subpopulations (T helper cells 1, Naive T helper cells 1, Memory T helper cells 1, T helper cells 2, Naive T helper cells 2, Memory T helper cells 2, Regulatory T cells) showed similar cell counts in both sexes.

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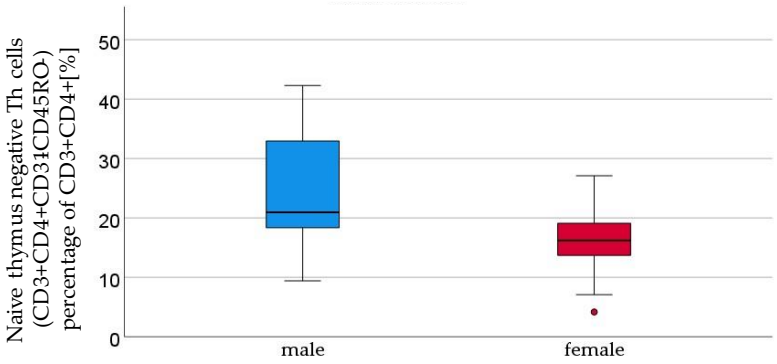

Figure 3. Thymus negative T lymphocytes (CD3+CD4+CD31-CD45RO-) percentage of CD3+CD4+ [%] were significantly higher in male (n=16) than in female (n=21) newborns (see Figure 1). Given with median (male = 21, female = 16.2), minimum (male 9.4, female 4.2) and maximum (male 42.3, female = 27.1); mean ± standard deviation [%] (male = 24.4 ± 9.8, female = 15.9 ± 5.7).

Table 2. Relative amounts of T lymphocyte subpopulations with mean and standard deviation [%]. Group differences were tested using Mann-Whitney U test.

	n	sex	Mean ± standard deviation [%]	p-value
T_h cells	16	male	74.4 ± 6.6	0.22
(percent of CD3+ cells)	21	female	76.8 ± 7.2	
Cytotoxic T cells	16	male	27.9 ± 7.6	0.12
(percent of CD3+ cells)	21	female	24.2 ± 7.7	
T_h cells with αβ-TCR CD4+CD6+	16	male	76.0 ± 6.4	0.5
(percent of TCRab+ cells)	21	female	77.3 ± 7.1	
Activated cytotoxic T_h-cells with αβ-TCR	16	male	0.6 ± 0.6	0.27
(percent of TCRab+ cells)	21	female	0.9 ± 0.8	
Memory effector T_h cells	16	male	49.5 ± 13.6	0.48
(percent of CD3+CD4+ cells)	21	female	52.1 ± 15.8	
Memory central T_h cells	16	male	5.7 ± 2.8	0.58
(percent of CD3+CD4+ cells)	21	female	6.7 ± 4.1	
Naive effector T_h cells	16	male	40.9 ± 15.2	0.17
(percent of CD3+CD4+ cells)	21	female	34.9 ± 11.8	
Naive central T_h cells	16	male	3.9 ± 1.4	0.62
(percent of CD3+CD4+ cells)	21	female	6.4 ± 9.4	
Memory effector cytotoxic T cells	16	male	50.8 ± 13.8	0.4
(percent of CD3+CD8+ cells)	21	female	53,.7 ± 14.1	
Memory central cytotoxic T cells	16	male	6.2 ± 4.3	0.66
(percent of CD3+CD8+ cells)	21	female	7.8 ± 6.4	
Naive effector cytotoxic T cells	16	male	39.4 ± 14.6	0.24
(percent of CD3+CD8+ cells)	21	female	34.1 ±1 0.6	

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Naive central cytotoxic T cells	16	male	3.6 ± 1.6	0.73
(percent of CD3+CD8+ cells)	21	female	4.5 ± 4.1	
Naïve thymus negative T_h cells	16	male	24.4 ± 9.8	0.005
(percent of CD3+CD4+ cells)	21	female	15.9 ± 5.7	
Naïve thymus positive T_h cells CD31+	16	male	65.7 ± 9.4	0.6
(percent of CD3+CD4+ cells)	21	female	70.8 ± 9.6	
T_h1 cells	11	male	2.8 ± 1.6	0.61
(percent of CD3+ cells)	16	female	2.8 ± 1.9	
Naive T_h1 cells	11	male	87.2 ± 7.0	0.54
(percent of CD3+ CD4+ CD183+ cells)	16	female	88.1 ± 6.8	
Memory T_h1 cells	11	male	12.8 ± 7.0	0.51
(percent of CD3+ CD4+ CD183+ cells)	16	female	11.9 ± 6.8	
T_h2 cells	11	male	0.7 ± 0.5	0.54
(percent of number of CD3+ cells)	16	female	0.5 ± 0.3	
Naive T_h2 cells	11	male	66.8 ± 8.6	0.9
(percent of CD3+ CD4+ CRTH2+ cells)	16	female	67.6 ± 11.2	
Memory T_h2 cells	11	male	33.2 ± 8.6	0.9
(percent of CD3+ CD4+ CRTH2+ cells)	16	female	32.4 ± 11.2	
Regulatory T cells	11	male	2.3 ± 0.6	0.8
(percent of CD3+ cells)	16	female	2.4 ± 0.7	

3. Discussion

In our study, we found significant differences in B and T cell populations between male and female newborns. We were able to generate reference values for nine B cell subpopulations and 21 T cell subpopulations, respectively.

Although sex related differences have been observed in children and adults, little is known about sex differences at birth. Research data about the latter are sparse and pathophysiologic pathways and mechanism remain elusive. The production of androgens in male fetus begins within the first trimenon at ten weeks of gestation marking an early initiation of sex differentiation also affecting the developing immune system. Sex differences in immune responses are traced back to two main factors: The influence of sex hormones as testosterone and oestrogen, and differences in the number of immune related genes on the X chromosome.[14]

The influence of immune related genes on X chromosome, leading to sex differences in the development of immune related diseases starts early in life. Here, umbilical cord blood allows a special insight into intrauterine lymphocyte profiles, as it represents the interphase between pre – and postnatal physiology. In our analysis, B cell populations were higher in male newborns compared to females, except for naive B cells and transitional B cells. These results are contrary to the relation of B cells in adult blood with higher B cell count in females than in male.[22] Possible reasons are changes of sex hormone levels *in utero* and in cord blood: Oestrogen stimulates the proliferation of B cells, while testosterone supresses.[15] After birth, there is a drastic increase of testosterone level in male newborns over the first three months, before dropping to a much lower pre-adolescence level at the age of 7-12 months. Females show a decrease of testosterone immediately after birth. Beyond the first year of life until adolescence, there are no sex specific differences in testosterone levels.[23],[14] This fact might lead to the assumption that sex hormones are less relevant during this period of life. Here, partially unknown factors, apart from sex chromosomes, might play an important role for the developing immune system.

Nevertheless, men are more prone to infection-induced inflammation[24], like respiratory tract infections (RTI). Being more susceptible to RTI than women are, severe courses of RTI infections are more often seen in male. This might be due to innate immune responses and its sex differences. A possible explanation is an observed imbalance of TLR-2 and TLR-4: there is a higher expression of TLR-4 in macrophages of male mice following endotoxic shock, leading to extensive production of

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pro-inflammatory cytokines.[25] A protective factor seems to be the expression of TLR-2, higher in female mice augmenting resistance against viral infections (especially coxsackie-virus).[26] Oestrogen on the other hand suppresses lung inflammation in animal models.[26] X-chromosome and its linked immune related genes as TLR 7/8 encoding genes might also be responsible for sex differences in infection-induced inflammation.[27] X disomy leads to a higher genetic variety in immune related genes. TLR 7/8 is able to detect viral single-stranded RNA (ss-RNA) and to induce protective cytokine responses, in particular IFN responses. Although male and female innate immune cells do not differ significantly on their overall TLR 7/8 expression, a humanised mouse model showed a positive influences on TLR7 ligation on the plasmacytoid dendritic cells and its IFN-α and TNF response.[28] Besides, micro-RNAs (mi-RNA) as non-coding RNAs are associated with inflammatory diseases. Loci of mi-RNA are to be found in a higher amount on X chromosomes than in Y-chromosomes and in autosomes.[28] X-linked miR223 seems to play a crucial role: studies suggest that miR223-negative mice showed more inflammatory symptoms compared to non-deficient mice following Candida albicans infection, miR223 is supposed to affect granulocyte generation and maturation negatively.[29,30]

Former research showed a postnatal increase of CD19+ B cells and CD3+ T cells, which remains stable until the age of 2 years, followed by a gradual decrease until adulthood. While CD3+CD4+ T lymphocytes comply this trend, the subpopulation of CD3+CD8+ T lymphocytes remains stable for the first two years of life followed by a constant decrease until reaching levels similar to adults.[31] Overall, no sex differences were considered in the aforementioned studies. Various influences on lymphocyte populations within this period, such as X chromosomal genes must be considered when analysing data on the developing immune system. There is less evidence on sex related changes in lymphocyte subpopulation and more research is necessary to illuminate all factors leading to the observed changes.

In vitro, estradiol increases the accumulation of B cells, but in healthy doses, it has no effect on proliferation response. Moreover, it displays the same effect on male and female lymphocytes. Non-toxic concentrations of testosterone do not influence the maturation of *in vitro* B cells.[1]

Sexual hormones also influence T cell subpopulations. Testosterone supresses the lymphopoesis only in early stages. After further development, T cells do not express androgen receptors. However, oestrogen receptors are expressed at any stage of the T cell development: low doses of oestrogen increase CD4+ cells, high doses of oestrogen reduce CD4 and CD8 cell counts.[16] In our analysis, four out of 14 T cell subpopulations (Cytotoxic T cells, naive effector T_h cells, naive effector cytotoxic T cells, naive thymus negative T_h cells) showed a trend to be higher in cord blood of male newborns. The remaining T cell subpopulations were expected to be higher in female newborns.

Even though there is much data about CD4+ and CD8+ T cell sex differences in general, there is still few evidence about sex differences in smaller lymphocyte subpopulations. We provide reverence values for 14 different T cell subpopulations. There is a need of further studies to gain more data about sex related differences in these subgroups. This could be important for identifying risk factors for diseases.

Regardless of sparse data on sex differences in immunity (or immune development) of neonates, published research indicates that male newborns are more prone to develop a robust innate immunity, characterised by higher counts of NK cells, monocytes and basophils. Male newborns additionally show a better pro inflammatory response compared to females.[17] However, females do have higher CD4+ T cells and CD4+:CD8+ ratios, lower CD8+ T cells and lower T_{reg} cell frequencies.[17] Numbers of B cells, IgG and IgM are similar in both sexes, whilst male newborns show higher IgA and IgE than females.[17] Our data suggest that there are no changes in the total population of B or T cells, but there are changes in their subpopulations. Subpopulations of B and T lymphocytes are very inhomogeneous groups that underlay different influences and react differently to certain stimulations. More studies are needed to identify factors leading to changes in lymphocyte subpopulations.[17]

Sex differences in immune response can be found throughout childhood. Male show higher inflammation and higher NK cell counts; CD4/CD8 ratios, CD8+ cells and CD4+ cells and B cell numbers are similar.[17],[33] Puberty marks a shift in sex differential immunity, which is potentially

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caused by sex steroids. Adult females show inflammation, CD4/CD8 ratios, CD4+ cells. While male adults show higher counts of CD8+ cells. B cells and immunoglobulin are higher in females. T_{reg} cells are higher in males.[17] After menopause, pro-inflammatory response in males is higher, while females show a higher T cell activation. Other sex differences as CD4+CD8+ cell count an CD4/CD8 ratio remain distributed equally.[17]

We found a significant difference in marginal zone B cells (p=0,025). Even though they only count 5% of all B cells, they play an important role in early immune response. MZ B cells are located at a strategic interface between blood and lymph nodes, so they are able to react promptly with antibody response and bacterial pathogens.[1] They close the gap between early immune response and late adaptive antibody reactions of follicular B cells.[8] A very interesting finding within our data marks the higher count of marginal zone B cells in male newborns. This finding is contradictory to published data, as females of all ages were found to show greater B cell numbers in total. The latter might be an example for changes in composition of lymphocyte populations after birth, which might lead to sex related differences in diseases in neonatal period and later in life.

B1 cells, defined as CD20+CD27+CD43+, also showed a significant difference (p=0.01). B1 cells are the main part of B lymphocytes in newborns, but reduce towards 10% until adulthood.[6] This illustrates that B1 cells are a dynamic part of the immune system, underlying constant changes during the development towards a "mature" immune response. B1 cells are an important part of innate immune system and have been investigated thoroughly. Yet some underlying pathomechanisms remain elusive.[33] Establishing a consistent definition of immune markers leads studies on B1 cell (sub)-populations to be more comparable which is of utmost importance. As a major finding of our study, higher count of B1 cells in male newborns were found. B1 cells are associated with the pathogenesis of asthma and other allergies, in particular with production of IgE antibodies, the regulation of inflammatory processes[34],[35] and the development of autoimmune diseases, mostly affecting women.[36] Our findings suggest changes in the composition of B1 cells after birth, which might be partially due to environmental influences, antigen exposure, or the development of the adaptive immune response.

Thymus negative T cells were significantly higher in male newborns (p=0,005). Innate T cells migrate from bone marrow to thymus, where they undergo a positive and negative selection. During positive selection process, T_h cells able to bind MHC complexes receive positive growing signal and entrance cell cycle. This positive selection takes place in the postnatal thymus, indicating that maturation of T_h cells is an ongoing process by the time of birth.[1]

Thymus negative T cells are innate cells, without completed positive and negative selection. After negative selection, mature T cells leaving thymus usually carry CD31 marker. Negative selection plays an important part in preventing development of autoimmune reactive cells. There are tissue specific antigens expressed in thymus. Autoimmune regulator genes (AIRE) control the expression of these antigens. Mutations in this gene may lead to an autoimmune disease called autoimmune polyglandular syndrome 1 (APS-1).[9] Therefore, changes in thymus negative T cell count could be involved in development of autoimmune diseases as APS-1. There are studies hinting correlation between a failure in negative thymus selection and rheumatoid arthritis.[37] Changes in thymus negative counts could induce dysregulations in the immune system leading to reduced or incorrect pathogen detection.

In conclusion, we found significant differences in cord blood lymphocytes, related to male and female neonates, respectively. Sex related differences undergo changes throughout the development towards a mature immune response. Multiple factors are responsible for sex differences in immunological responses throughout life. Pro-inflammatory responses follow changes in puberty and post-menopause indicating oestrogen influence. Other factors remain constant from birth to adulthood. By establishing reference values for nine B cell and 21 T cell subpopulations in cord blood, our study provides important data on the influence of biological sex in the developing immune system. Investigation of influencing factors, such as genetic conditions and sexual hormones after birth are important for a deeper comprehension of early immune development and its sex related differences in neonates, infants, children and young adults.

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4. Materials and Methods

4.1. Patient samples

We collected blood samples from umbilical cord blood of term neonates (range 37-40 weeks gestation, n=37) without any infectious, immunologic or chronical disease or antibiotic treatment in the previous two weeks, before planned caesarean section. The Ethics Committee of Saarland approved the study protocol (198/20, 06.08.2020). The written informed consent of the parents was obtained.

4.2. Cell isolation, storage and counting

Cord blood mononuclear cells (CBMC) were isolated by density gradient centrifugation using Lymphocyte Separation Medium 1077. (Linaris biological products, Dossenheim, Germany). Subsequently, CBMCs were stored in FCS+10% DSMO in cryoconservation at a temperature of -80 °C. Prior to the cell analysis, the cryopreserved cells were gradually thawed by first acclimating the frozen vial on ice and second thawing the cell pellet in a water bath at room temperature. Pellets were washed using PBS (Sigma Aldrich, Steinheim, Germany) and stained with Acridinorange/Propidiumiodid (AO/PI) (Logos Biosystems, Gyeonggi-do 14055 South Korea) before they were counted by LUNA-FL™ Automated Fluorescence Cell Counter (Logos Biosystems, Gyeonggi-do 14055 South Korea) to determine the number of viable cells from the donor.

4.3. Flow cytometry

To quantify the lymphocyte subsets, cells were labelled according to table 4. B lymphocytes were marked with anti-CD5, anti-CD19, anti-CD20, anti-CD27, anti-CD38, anti-CD43, anti-IgM and anti-IgD, T lymphocytes with anti-CD3, anti-CD4, anti-CD8, anti-TCR αβ+, anti-CD62L, anti-CD69, anti-CD45RO anti-CD31, anti-CCR4, anti-CCR6, anti-CRTH2, anti-CD25, anti-CD127 and anti-CD183, respectively.

BD Horizon Fixable Viability Stain 780 (Becton, Dickinson & Company, Heidelberg, Germany) was used for was subsequent discrimination of dead cells. After incubation protected from light, followed by washing steps, cell suspension was partitioned and prepared for cell staining. Cell pellets were resuspended in panel specific antibody mix and incubated protected from light at room temperature for 30 minutes. Remaining Erythrocytes were lysed with BD FACS™ Lysing Solution (Becton, Dickinson & Company, Heidelberg, Germany) according to the manufacturers protocol. After centrifugation, the supernatant was discarded, and pellets were resuspended in PBS and stored in a light-protected manner on ice until measurement.

Flow cytometry was performed on a three laser FACS Celesta flow cytometer (Becton, Dickinson & Company, Heidelberg, Germany). By forward scatter-high (FSC-H) plotted against forward scatter-area (FSC-A) doublets and cell aggregates were excluded (1.2). Gates were preset and the measurements were performed blinded for sample identity. Dead cells were excluded from the analysis by staining with BD Horizon Fixable Viability Stain 780.

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Table 3. Surface markers matched with Fluorophores and clones.

marker	fluorophore	clone
CD127	**Alexa-Fluor 647**	HIL-7R-M21
CD183	BV480	CXCR3
CD19	APC-R700	HIB19
CD194 (CCR4)	PE	1G1
CD196 (CCR6)	APC-R700	CCR6
CD20	BB700	2H7
CD21	BV421	B-Ly4
CD25	BV421	M-A251
CD27	APC	L128
CD294 (CRTH2)	BV650	BM16
CD3	BV786	SK7
CD31	BV421	L133.1
CD38	PE	HB-7
CD4	PE-CF594	SK3
CD43	BV605	1G10
CD45RO	BV605	UCHL1
CD5	BV650	L17F12
CD62L	BB700	SK11
CD69	BV480	FN50
CD8	APC-R700	SK1
IgD	PE-CF594	IA6-2
IgM	BV480	G20-127
TCRαβ	FITC	WT31
TCRγδ	PE	11F2

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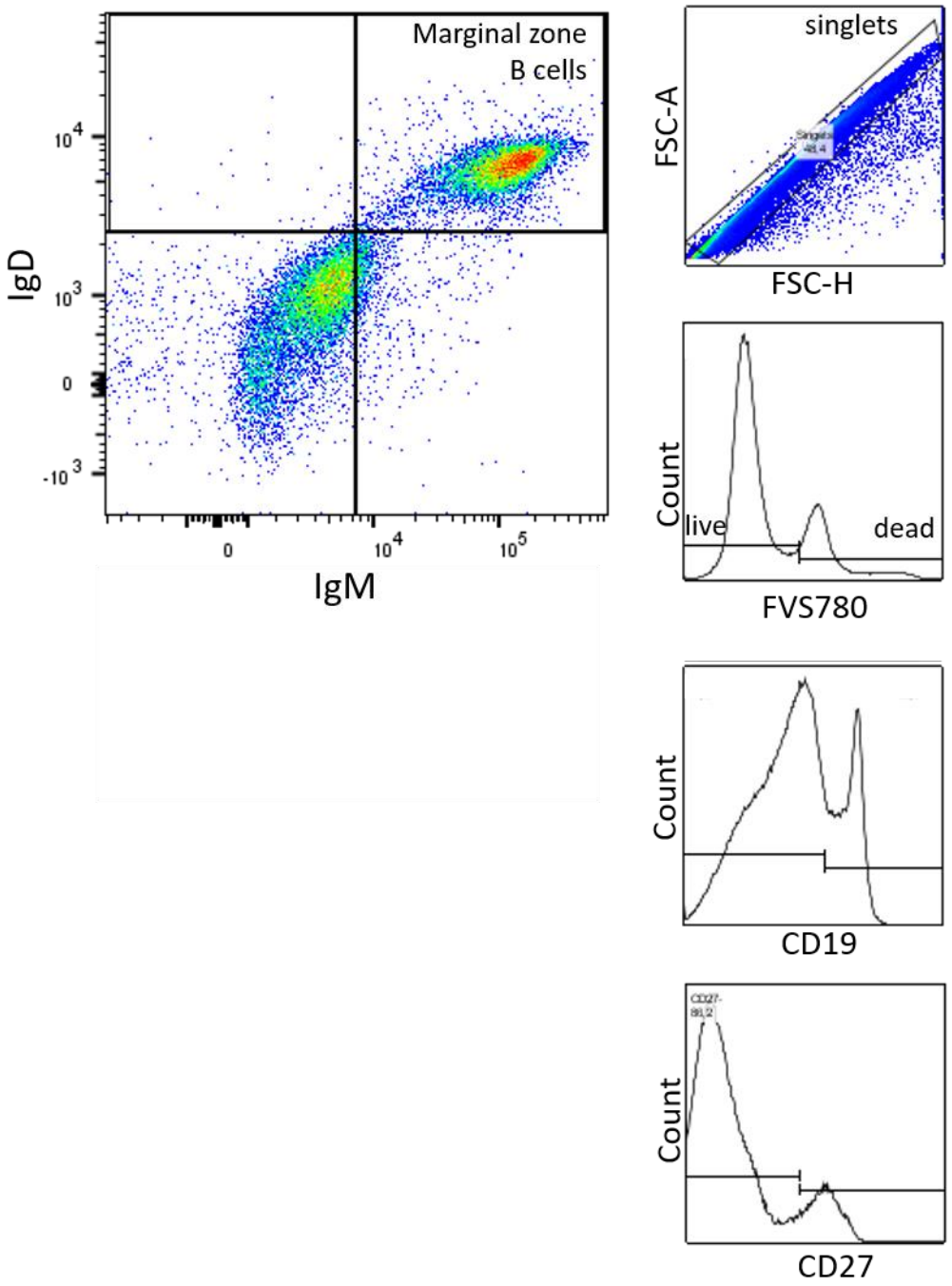

Figure 4. Gating Strategy of Marginal zone B Cells: 1.1 FSC-H x FSC-A. 1.2. Histogramm of Comp-Cy7-A x FVS780. 1.3 Histogramm of Comp-PerCP-Cy5-5-A x CD20. 1.4. Histogramm of Comp-APC-R700-A x CD27. 1.5.Comp-PE-CF594-A: IgD x Comp-BV510-A: IgM.

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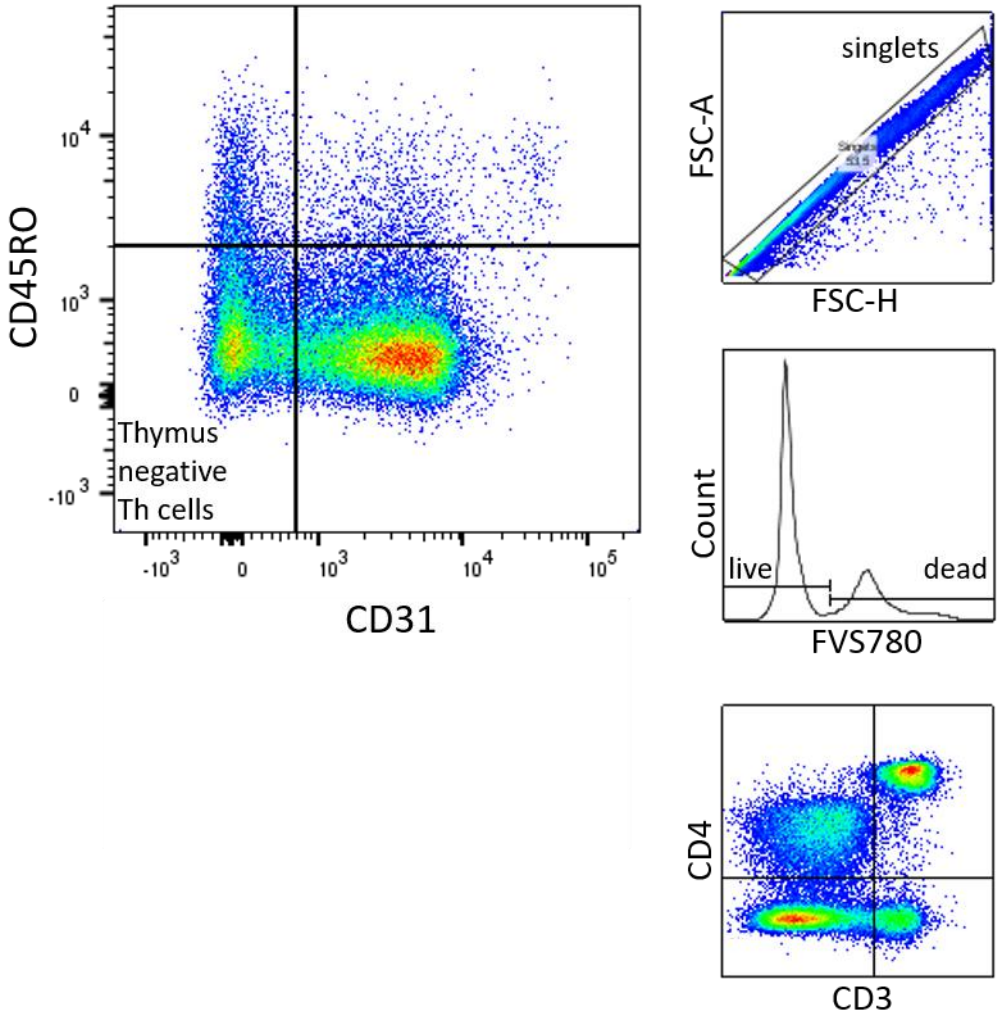

Figure 5. Gating Strategy of B1 Cells: 1.1 FSC-H x FSC-A 1.2. Histogramm of Comp-Cy7-A x FVS780. 1.3. Histogramm of Comp-PerCP-Cy5-5-A x CD20. 1.4. Histogramm of Comp-APC-A x CD27. 1.5.SSC-A x Comp-BV605-A:CD43.

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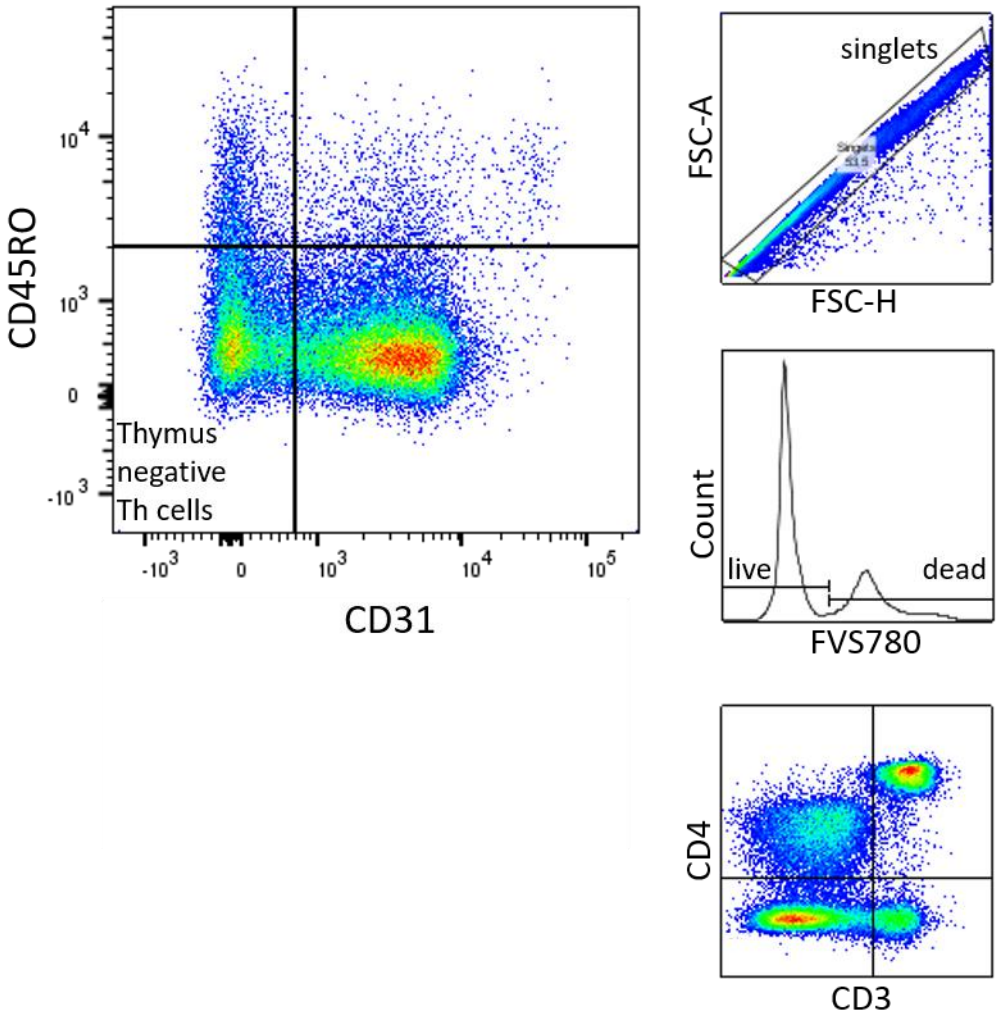

Figure 6: Gating Strategy of naive thymus negative T Cells: 1.1 FSC-A x FSC-H. 1.2. Histogramm of Count x FVS780. 1.3. CD4 x CD3 1.4. CD45RO x CD31.

Table 4. Definition of B and T lymphocyte subpopulations. Surface markers for 11 B and T lymphocytes.

Lymphocyte subsets		Definition
B lymphocytes	B cells	CD19+
	Innate B cells	CD19+CD27-IgD-IgM-
	Naive B cells	CD19+CD27-IgD+IgM+
	Memory B1 cells	CD19+CD27+
	Marginal zone memory B cells	CD19+CD27+IgD+IgM+
	Class switched memory B cells	CD19+CD27+IgD-IgM-
	Late memory B cells	CD19+CD27+CD38+IgM+
	Plasmablasts	CD19+CD27+CD38++IgM-
	Transitional B cells	CD19+CD20+CD27-CD38+
	B1 cells	CD20+CD27+CD43+
	B2 cells	CD20+CD27+CD43-
T lymphocytes	T cells	CD3+
	T helper cells	CD3+CD4+
	Cytotoxic T cells	CD3+CD8+
	T helper cells with αβ-TCR	TCRαβ+CD4+

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Cytotoxic T helper cells with αβ-TCR	TCRαβ+CD8+
Activated T helper cells αβ-TCR	TCRαβ+CD4+CD69+
Activated cytotoxic T helper cells with αβ-TCR	TZR αβ+ CD8+CD69+
Memory effector T helper cells	CD3+CD4+CD62L-CD45RO+
Naive central T helper cells	CD3+CD4+CD62L+CD45RO+
Naive effector T helper cells	CD3+CD4+CD62L-CD45RO-
Naive central T helper cells	CD3+CD4+CD62L+CD45RO-
Memory effector cytotoxic T cells	CD3+CD8+CD62L-CD45RO+
Memory central cytotoxic T cells	CD3+CD8+CD62L+CD45RO+
Naive effector cytotoxic T cells	CD3+CD8+CD62L-CD45RO-
Naive central cytotoxic T cells	CD3+CD8+CD62L+CD45RO-
Naive thymus negative T helper cells	CD3+CD4+CD31-CD45RO-
Naive thymus positive T helper cells	CD3+CD4+CD31+CD45RO-
T helper cells 1	CD3+CD4+CD183+CCR6+
Naive T helper cells 1	CD3+CD4+CD183+CD45RO-
Memory T helper cells 1	CD3+CD4 CD183+CD45RO+
T helper cells 2	CD3+CD4+CCR4+CRTH2+
Naive T helper cells 2	CD3+CD4+CD45RO-CRTH2+
Memory T helper cells 2	CD3+CD4+CD45RO+CRTH2+
Regulatory T cells	CD3+CD4+CD25+CD127-

4.4. Statistical Analysis

Statistical analysis was performed using SPSS 28.0.0. (IBM, Chicago, USA). Group differences were tested using Mann-Whitney U Test assuming non-normally distributed data. Differences with p-values of $p < 0.05$ were deemed significant. Means are given with standard deviation (SD). Boxplots in the figures show 10 to 90 percentile (whiskers).

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions:

MB: conception and design, coordination of sample collection, data analysis and interpretation, manuscript writing, final approval of manuscript.

CS: collection and assembly of data, manuscript writing, final approval of manuscript.

ST: coordination of sample collection, manuscript writing, final approval of manuscript.

BH: manuscript writing: final approval of manuscript.

RW: MH: collection and assembly of data, data analysis, manuscript writing, final approval of manuscript.

EK: collection and assembly of data, manuscript writing

GW: data analysis and interpretation, manuscript writing, final approval of manuscript.

NNT: manuscript writing: final approval of manuscript.

MZ: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript.

SGF: conception and design, coordination of sample collection, data analysis and interpretation, manuscript writing, final approval of manuscript.

All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Saarland University (198/20, 06.08.2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest

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