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The Hypoxia Affects the Main Thymocyte Subset Distributions in Congenital Heart Diseases

Hipoksi Konjenital Kalp Hastalıklarında Ana Timosit Alt Grup Dağılımlarını Etkilemektedir

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Abstract

Objective: Congenital heart diseases (CHD) are often associated with thymus development disorders and immune dysfunctions. However, the features of thymocyte differentiation in cyanotic and acyanotic CHD remain unknown.

Materials and Methods: We have analyzed the main thymocyte subsets depending on CD4 and CD8 co-expression, and the number of natural regulatory T-cells (nTreg) and invariant natural killer T-cells (iNKT) precursors in the co-cultures of thymocytes with thymic plasmacytoid (p) dendritic cells (DCs) *in vitro*, isolated from the thymus of children with acyanotic (without hypoxia) and cyanotic (with severe hypoxia) CHD.

Results: In the thymocyte co-cultures with pDCs in cyanotic CHD, compared to acyanotic CHD, a decreased number of thymocytes expressing αβ chains of the T-cell receptor with CD4 and CD8 lower levels ($CD4^{lo}CD8^{lo}\alpha\beta TCR^{+}$), but the increased numbers of $CD4^{hi}CD8^{-/lo}\alpha\beta TCR^{+}$ cells were detected. The numbers of $CD4^{-}CD8^{-}\alpha\beta TCR^{+}$, $CD4^{hi}CD8^{hi}\alpha\beta TCR^{+}$, $CD4^{-/lo}CD8^{hi}\alpha\beta TCR^{+}$ cells did not differ between cyanotic or acyanotic CHD. In cyanotic CHD in the thymocyte co-cultures with pDCs, the decreased number of $CD4^{+}CD25^{+}FOXP3^{+}$ cells, nTreg precursors, was detected in comparison with acyanotic CHD. In cyanotic CHD, the number of $CD3^{hi}V\alpha24J\alpha18^{+}$ cells, iNKT precursors, in the thymocyte co-cultures with pDCs did not differ in comparison with acyanotic CHD. Hypoxia in cyanotic CHD increased the resistance to apoptosis of thymocytes in co-cultures with pDCs in comparison with acyanotic CHD.

Conclusion: Thus, hypoxia affected the main $CD4^{+}$ and $CD8^{+}$ αβTCR T-cell subsets and the number of $CD4^{+}CD25^{+}FOXP3^{+}$ cells in the thymocyte co-cultures with pDCs isolated from thymus of children with CHD.

Keywords: Congenital heart diseases, hypoxia, thymocytes, iNKT, nTreg, pDCs

Öz

Amaç: Konjenital kalp hastalıkları (KKH) sıklıkla timus gelişim bozuklukları ve immün fonksiyon bozuklukları ile ilişkilidir. Bununla birlikte, siyanotik ve asiyanotik KKH'de timosit farklılaşmasının özellikleri halen bilinmemektedir.

Gereç ve Yöntem: CD4 ve CD8 ko-ekspresyonuna bağlı olarak asiyanotik (hipoksisiz) ve siyanotik (şiddetli hipoksili) KKH'li çocukların timüsünden izole edilen, ana timosit alt kümelerini ve timik plazmasitoid (p) dendritik hücreler (DH'ler) ile timositlerin ko-kültürlerinde doğal regülatör T-hücreleri (nTreg) ve değişmez doğal öldürücü T-hücreleri (iNKT) prekürsörlerinin miktarlarını *in vitro* olarak analiz edildi.

Bulgular: Siyanotik KKH'de pDH'li timosit ko-kültürlerinde, asiyanotik ile karşılaştırıldığında, daha düşük seviyelerde CD4 ve CD8 ile T-hücresi reseptörünün (THR) αβ zincirlerini eksprese eden timosit sayısında azalma tespit edildi ($CD4^{lo}CD8^{lo}\alpha\beta THR^{+}$), ancak artan sayıda $CD4^{hi}CD8^{-/lo}\alpha\beta THR^{+}$ hücreleri bulundu. $CD4^{-}CD8^{-}\alpha\beta THR^{+}$, $CD4^{hi}CD8^{hi}\alpha\beta THR^{+}$, $CD4^{-/lo}CD8^{hi}\alpha\beta THR^{+}$ hücrelerinin sayısı açısından siyanotik veya asiyanotik KKH arasında farklılık görülmedi. pDH'li timosit ko-kültürlerinde siyanotik KKH'de, asiyanotik KKH ile karşılaştırıldığında $CD4^{+}CD25^{+}FOXP3^{+}$ hücrelerinin, nTreg prekürsörlerinin sayısında azalma tespit edildi. Siyanotik KKH'de, pDH'li timosit ko-kültürlerinde iNKT prekürsörleri olan

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$CD3^{hi}V\alpha24J\alpha18^{+}$ hücrelerinin sayısı, asiyanotik KKH ile karşılaştırıldığında farklılık göstermedi. Siyanotik KKH'deki hipoksi, asiyanotik KKH'ye kıyasla pDH'li ko-kültürlerde timositlerin apoptoza direncini artırdı.

Sonuç: Bu nedenle hipoksi, KKH'li çocukların timüsünden izole edilen pDH'ler ile timosit ko-kültürlerindeki ana CD4 ve CD8 αβTHR T-hücresi alt kümelerini ve $CD4^{+}CD25^{+}FOXP3^{+}$ hücrelerinin sayısını etkilemiştir.

Anahtar Kelimeler: Konjenital kalp hastalıkları, hipoksi, timositler, iNKT, nTreg, pDH'ler

Introduction

Congenital heart diseases (CHDs) are one of the most common developmental abnormalities in children and are significant causes of immunity disorders (1-3). CHDs are often associated with thymic dervelopmental disorders, since the cells of the cranial neural crest involved in the formation of the heart connective tissues also participate in thymic stroma formation (1-3). Multiple morphological and functional changes in the thymus revealed in children with CHD affect the thymocyte differentiation (1-3). Hypoxia is one of the most common complications in CHD due to changes in systemic hemodynamics (4). The severity of hypoxia in CHD is classified according to the clinical manifestations of mucous cyanosis: Acyanotic defects without hypoxia (with left-right blood shunt without mixing arterial and venous blood); cyanotic defects with severe hypoxia (with right-left blood shunt with mixing of arterial and venous blood) (4,5). Hypoxia causes stereotypical changes in cells associated with lack of oxygen, energy deficiency, and an anabolism decrease (4,5). Changes in the number and structure of mitochondria and the synthetic apparatus of thymocytes are revealed in hypoxia in CHD (3-5). Structural changes in reticular epithelial cells depending on the severity of CHD and hypoxia inhibit thymocyte proliferation and development during positive selection (3-6). The numbers of recent migrants from the thymus and T-lymphocyte absolute number are significantly reduced in the peripheral blood of children with CHD (7). T-lymphocyte subpopulation compositions, proliferation and cytokine production are also changed (1-3,8). In addition, autoimmune and atopic disorder frequency is increased in children with CHD, apparently, due to natural regulatory T-cell (nTreg), invariant natural killer T-cells (iNKT), and other regulatory cell dysfunctions (1,5). A few studies have shown that the number of nTregs in peripheral blood decreased in children with CHD (1,5). We previously established that hypoxia in CHD affects both the cytokine-induced expression of FOXP3 (forkhead box P3) and RORC-γt (retinoic acid-related orphan receptor γt) transcription factors in $CD4^{+}$ thymocytes and the formation of nTreg and natural interleukin-17 producing T helpers precursors *in vitro* (9). Thymic dendritic cells (DCs) together with reticular epithelial cells play a leading role in thymocyte differentiation, division into $CD4^{+}$ and $CD8^{+}$ subpopulations, and nTreg and iNKT precursor developments (10). Our previous histological studies have shown that immature plasmacytoid (p) DCs predominated in the thymus medulla and cortex, depending on the severity of CHD and hypoxia (3,9,11). However, the thymocyte differentiation with the presence of thymic pDCs in CHD with hypoxia is not fully understood, namely for main thymocyte subsets, expressing αβ chains of the T-cell receptor (αβTCR), nTreg, and iNKT. Therefore, here, we sought to investigate the thymocyte differentiation in the presence of pDCs isolated from thymus of children in cyanotic or acyanotic CHD, covering main αβTCR T-cell subsets, nTreg, and iNKT *in vitro*.

Materials and Methods

Patient Samples

This study was approved by the local Ethics Committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences in accordance with the Declaration of Helsinki. Thymus fragments were obtained from the children under 1 year of age, undergoing cardiothoracic surgery due to CHD at the Federal Center for Cardiovascular Surgery named after S. G. Sukhanov, Ministry of Health of Russia. The children had no known genetic abnormalities. Written informed consents were obtained from the patients' parents. A thymectomy was performed according to the present surgical practice. Division into the groups of acyanotic (without hypoxia and cyanosis of the mucous membranes and skin) and cyanotic (with pronounced hypoxia and cyanosis of the mucous membranes and skin) CHD was performed considering the diagnosis (when analyzing the data of medical records) and the severity of hypoxia and cyanosis. The study included 19 thymi (9 in acyanotic CHD; 10 in cyanotic CHD).

Cell Isolation and *in vitro* Culture

Thymus fragments were placed in culture medium (RPMI-1640 with GlutaMAX™ -I (Gibco®, UK) supplemented with penicillin 50 mU/mL and streptomycin 50 ng/mL, 10% fetal bovine serum, 25 mM HEPES (N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid, Gibco®, UK) and cut into small pieces. The thymocytes were released into the culture medium by pressing the tissue lightly.

pDCs were sorted from the same thymus using immunomagnetic isolation kits (Diamond plasmacytoid dendritic cells isolation kit II, Human, Miltenyi Biotec, Germany) according to the manufacturer's instructions. Preliminarily, thymic tissue was mechanically disrupted and obtained suspension was concentrated by centrifugation in ficoll-verografin density gradient 1.077 g/cm^3, followed by the filter cell suspension using a 30 μm cell strainer (Miltenyi Biotec, Germany) to remove debris. Cells from the low density fraction were used for DC isolation (2×10^8 cells/mL). The purity of the isolated DCs verified by flow cytometry with monoclonal antibodies (mAbs) (BioLegend, USA) to CD303 (anti-human CD303 (BDCA-2) FITC, clone 201A) (10) was typically more than 90% (Supplement Figure 1B). For reaggregated culture, purified pDCs were mixed with autologous thymocytes in ratio of 1:10 (1×10^5:1×10^6 cells/mL) in U-bottom 96-well plates, centrifuged at 800 rpm for 5 min, and incubated together for 72h, at 37°C under 5% CO_2. After incubation, the aggregates were harvested, washed and stained with mAbs for phenotype analysis by flow cytometry. Supernatants were collected. Cell viability after 72h cultivation was determined by trypan blue exclusion test. The number of viable cells recovered from these co-cultures did not differ between cyanotic and acyanotic CHD (number of thymocytes (M ± m) in co-cultures with pDCs in cyanotic CHD - (0.75 ± 0.18) ×10^6/mL; in acyanotic CHD: (0.61 ± 0.12) ×10^6/mL). The design of experiments was presented in Supplementary information (Supplement Figure 1A).

Flow Cytometry

Flow cytometry was performed on a CytoFlex S flow cytometer using CytExpert and Kaluza 1.5 software (Beckman Coulter, USA). Total viable cells were enumerated using a restricted gate defined by forward versus side scatter (FSC/SSC) parameters and verified by the absence of propidium iodide (PI) incorporation (Figure 1A, H). All analyses were performed for viable cells. The thymocytes were distinguished from pDCs based on FSC/SSC parameters (Figure 1). The expression of CD4 (anti-human CD4 PE, clone RPA-T4, BioLegend, USA) and CD8 (anti-human CD8α PerCP/Cy5.5, clone RPA-T8, BioLegend, USA) on thymocytes expressing T-cell receptor αβ-chain (αβTCR) (FITC anti-human TCRαβ, clone IP26, BioLegend, USA) (Figure 1D, K) was determined (12-16). nTreg precursors were estimated as FOXP3$^+$cell percentage in gated CD4$^+$CD25$^+$thymocytes [Human CD4$^+$(FITC) CD25$^+$(PE) regulatory T-cell staining reagent, R&D, USA; anti-human FOXP3 PerCP/Cy5.5, clone PCH101, eBioscience, USA] (Figure 1F, M). iNKT precursors were detected as CD3^{hi}Vα24Jα18$^+$cell percentage in the thymocyte gate (anti-human CD3 PE-Cy5, clone UCHT1, eBioscience, USA; anti-human Vα24Jα18 TCR PE, clone 6B11, eBioscience, USA) (Figure 1G, N). Appropriate isotype-matched control antibodies were included for each analysis (Supplement Figure 2). Thymocytes were additionally stained with Annexin V-FITC and PI (Annexin V-FITC Kit, IM3546, Beckman Coulter, France) to determine the proportion of apoptotic cells and the viable cells (Supplement Figure 3) (15). In some co-cultures, 10^{-7} M dexamethasone (KRKA, Croatia) was added 16h before to the cultivation end for apoptosis induction. To detect intracellular Ki-67 and Bcl-2 expression, thymocytes were additionally stained for Bcl-2 (Brilliant Violet 421™ anti-Bcl-2 (clone 100, isotype mouse IgG1) or for Ki-67 (Brilliant Violet 421™ anti-human Ki-67 (clone Ki-67, isotype mouse IgG1, κ) both from BioLegend, USA) expressions. Cells were fixed and permeabilized in Fix&Perm cell permeabilization reagents (BioLegend, USA) according to the manufacturer recommendations. Gating strategy is presented in Supplementary information (Supplement Figure 4).

Cytokine Productions

The levels of IL-12 (p40) and IL-10 cytokines in the pDCs culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) commercial kits: IL-12 (p40) (ELISA MAX™ Deluxe SET Human IL-12/IL-23 (p40), BioLegend, USA) and IL-10 (interleukin-10-ELISA-BEST, Vector-BEST, Russia) using a microplate reader Synergy H1 (Bio-Tek Instruments, Inc., USA).

Statistical Analysis

The data were presented as median and the lower and upper quartiles, Me (LQ; UQ). Statistical analyses were performed using "STATISTICA 11.0" (StatSoft, USA). The Kolmogorov-Smirnov test was used for verifying normal distribution. The significance of differences between the groups of cyanotic and acyanotic CHD was assessed by t-test for independent samples (groups). The Spearman rank correlation (r) test was used for correlation analysis. The differences were considered significant at $p<0.05$.

Results

Features of CD4 and CD8 Expression by aβTCR$^+$ Thymocytes in Co-Cultures with pDCs in Acyanotic and Cyanotic CHD

Freshly isolated thymocytes had a predominantly immature αβTCR^{low} phenotype (Figure 1C) and contained double negative (DN) (CD4$^-$CD8$^-$), double positive (DP) (CD4$^+$CD8$^+$), single positive (SP) CD4 (CD4$^+$CD8$^-$) and CD8 (CD4$^-$CD8$^+$) αβTCR$^+$ thymocytes (Figure 1D). The phenotype conversion from a αβTCR^{low} to a more mature αβTCR$^{intermediate/high}$ phenotype was occurring during culturing with pDCs *in vitro* regardless of hypoxia (Figure 1C, J; Supplement Table 1). DP αβTCR$^+$ cells

consisted of cells with high expression of CD4 and CD8 ($CD4^{hi}CD8^{hi}\alpha\beta TCR^{+}$) and the intermediate/low $CD4^{lo}CD8^{lo}\alpha\beta TCR^{+}$ phenotypes after 72h co-cultivation with pDCs in both cyanotic and acyanotic CHD (Figure 1K). The $CD4^{lo}CD8^{lo}\alpha\beta TCR^{intermediate/high}$ phenotype is typical for thymocytes beginning positive selection (13). A decreased number of $CD4^{lo}CD8^{lo}\alpha\beta TCR^{+}$ cells was detected in thymocyte co-cultures with pDCs in cyanotic compared to acyanotic CHD, while $CD4^{hi}CD8^{hi}\alpha\beta TCR^{+}$ cell amount did not change (Figure 2). The number of $CD4^{lo}CD8^{lo}\alpha\beta TCR^{+}$ cells in cyanotic CHD decreased proportionally to $CD4^{hi}CD8^{-/lo}\alpha\beta TCR^{+}$ thymocytes, which was confirmed by the correlation analysis (r=-0.78; p=0.04) (Figure 2). We suggested that $CD4^{lo}CD8^{lo}\alpha\beta TCR^{+}$cell number reduced due to their differentiation into $CD4^{hi}CD8^{-/lo}\alpha\beta TCR^{+}$ cells in thymocyte co-cultures with thymic pDCs in cyanotic CHD. According to literature, DP thymocytes undergo apoptosis during development (15); therefore, we have examined the thymocyte apoptosis after 72h cultivation with pDCs (Table 1). The thymocytes in co-cultures with pDCs in cyanotic CHD were more resistance to apoptosis compared to acyanotic CHD (Table 1). The reduced thymocyte number entered into late (AnV^{+}/PI^{+}) apoptosis or necrosis was revealed in co-cultures with pDCs in cyanotic CHD compared to acyanotic CHD (Table 1). At the same time, there were no significant differences in the number of early (AnV^{+}/PI^{-}) apoptosis. Apoptosis resistance was accompanied by up-regulation of Bcl-2 expression in $\alpha\beta TCR^{+}$ thymocytes and also in $CD4^{+}$thymocytes cultivated with pDCs in cyanotic CHD compared to acyanotic CHD in dexamethasone treated co-cultures (Table 1). In dexamethasone untreated co-cultures, the percentage of $Bcl\text{-}2^{+}\alpha\beta TCR^{+}$ thymocytes and $Bcl\text{-}2^{+}CD4^{+}$thymocytes after 72h incubation with pDCs in cyanotic CHD increased slightly without significance compared to that of acyanotic CHD, which is attributed to small sample size of the study. Moreover, we investigated the Ki-67 expression, a molecule associated with cellular proliferation, in $\alpha\beta TCR^{+}$ thymocytes and also in $CD4^{+}$thymocytes (Supplement Table 2). There were no significant changes in the percentage of $Ki\text{-}67^{+}\alpha\beta TCR^{+}$thymocytes and $Ki\text{-}67^{+}CD4^{+}$thymocytes between cyanotic and acyanotic CHD. The results showed that hypoxia in cyanotic CHD affected the main CD4 and CD8 αβTCR T-cell subsets and apoptosis resistance.

Evaluation of $CD4^{+}CD25^{+}FOXP3^{+}$ Cells, Precursors of nTreg, in Co-Cultures of Thymocytes with pDCs in Acyanotic and Cyanotic CHD

Freshly isolated thymocytes contained less than 1% $CD25^{+}FOXP3^{+}CD4^{+}$cells (Figure 1F, Supplement Table 3). $CD4^{+}CD25^{+}FOXP3^{+}$ cell number did not significantly change after 72h incubation without pDCs (Supplement Table 3). Incubation of thymocytes with pDCs during 72h enhanced $CD4^{+}CD25^{+}FOXP3^{+}$ cell number (Figure 1M; Supplement Table 3). These data support an important role of pDCs in the generation of human nTreg. However, $CD4^{+}CD25^{+}FOXP3^{+}$ cell number was lower in the thymocyte co-cultures with pDCs in cyanotic CHD than that in acyanotic CHD (Figure 3). The Bcl-2 expression in $CD4^{+}CD25^{+}FOXP3^{+}$ cells increased slightly without significance after 72h incubation of thymocytes with pDCs in cyanotic compared to that in acyanotic CHD (Table 1). There were no significant differences in terms of $Ki\text{-}67^{+}CD4^{+}CD25^{+}FOXP3^{+}$cell percentage in co-cultures with pDCs in cyanotic as well as acyanotic CHD (Supplement Table 2). We have shown that, pDCs synthesized higher IL-12 (p40) level and lower IL-10 level in cyanotic compared to that in acyanotic CHD, but without statistical significance (Supplement Figure 3). Also, direct correlation between IL-10 concentration and $CD4^{+}CD25^{+}FOXP3^{+}$ cell number (r=0.75; p=0.04) in the thymocyte co-cultures with pDCs in acyanotic CHD was revealed. The obtained data supported that hypoxia in cyanotic CHD depressed $CD4^{+}CD25^{+}FOXP3^{+}$ cell formation in the presence of thymic pDCs *in vitro*.

Evaluation of the Number of $CD3^{hi}V\alpha24J\alpha18^{+}$ Cells, Precursors of the iNKT, in Co-Cultures of Thymocytes with pDCs in Acyanotic and Cyanotic CHD

Freshly isolated thymocytes contained less than 2% $CD3^{hi}V\alpha24J\alpha18^{+}$ cells (iNKT precursors) from $CD3^{hi}$thymocytes (Figure 1G) and their number did not significantly change after 72h incubation (Supplement Table 3). The thymocyte incubations during 72h with pDCs enhanced $CD3^{hi}V\alpha24J\alpha18^{+}$ cell numbers (Figure 1N, Supplement Table 3). These data supported pDCs important role in human iNKT generation in thymus. At the same time, there were no significant differences in the number of $CD3^{hi}V\alpha24J\alpha18^{+}$ cells in thymocyte co-cultures with pDCs between cyanotic and acyanotic CHD (Figure 3). The percentage of $Bcl\text{-}2^{+}CD3^{hi}V\alpha24J\alpha18^{+}$ cells was significantly higher in thymocytes after 72h incubation with pDCs in dexamethasone treated co-cultures in cyanotic compared to that in acyanotic CHD (Table 1). In dexamethasone untreated co-cultures, the percentage of $Bcl\text{-}2^{+}CD3^{hi}V\alpha24J\alpha18^{+}$ cells after 72h incubation with pDCs in cyanotic CHD increased slightly without significance compared to that of acyanotic CHD, but the number of $Ki\text{-}67^{+}CD3^{hi}V\alpha24J\alpha18^{+}$ cells had a tendency to decrease compared to the acyanotic CHD. Thus, the thymocyte development during hypoxia in cyanotic CHD did not affect the $CD3^{hi}V\alpha24J\alpha18^{+}$ cell number in the presence of thymic pDCs but enhanced Bcl-2 expression in iNKT precursors *in vitro*.

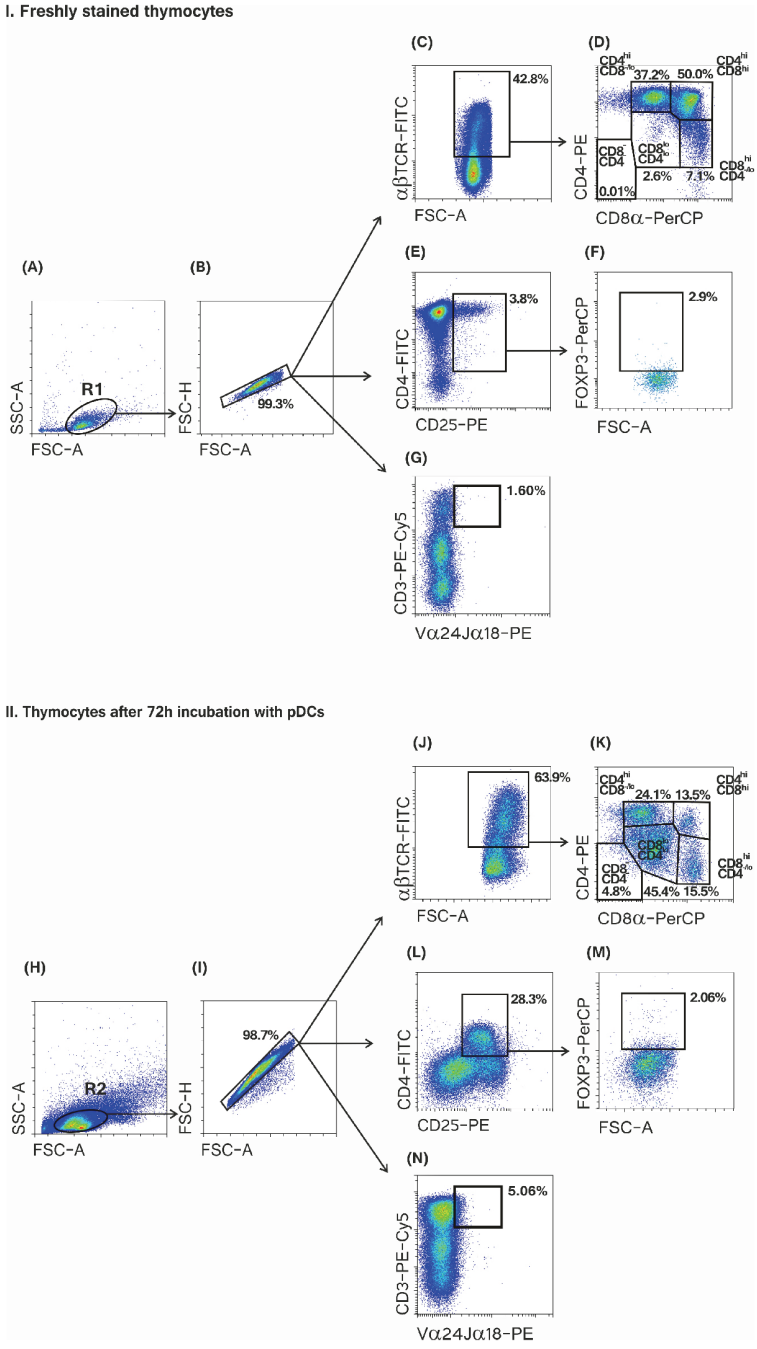

Figure 1. Flow cytometry analysis of freshly unfractionated thymocytes isolated from thymus (I) and unfractionated thymocytes co-cultured for 72h with the plasmacytoid dendritic cells (pDCs) (II low). The gating strategy depicted here was used for analyzing the main thymocyte subsets, expressing αβ chains of T-cell receptor (αβTCR$^+$ thymocyte), natural regulatory T-lymphocytes (nTreg), invariant natural killer T-cells (iNKT) in unfractionated thymocyte (R1) and after 72h co-cultivation with pDCs (R2).

Representative dot plots showed the gate used to identify the thymocytes (R1 for freshly isolated thymocytes (A); R2 for thymocytes after co-cultivation for 72h with the pDCs (H) based on FSC/SSC); next step for identification of viable cells and gated out of doublets and dead cells (B, I); αβTCR$^+$ thymocytes were identified by αβTCR expression (C, J) and the different thymocyte subsets were further gated (D, K) based on the low and high expression of CD4 and CD8 markers (CD4$^-$CD8$^-$; CD4^{lo}CD8^{lo}; CD4^{hi}CD8^{hi}; CD4^{hi}CD8$^{-/lo}$; CD4$^{-/lo}$CD8^{hi}). nTreg were defined within CD4$^+$CD25$^+$thymocytes (E, L) in R1 and expressing FOXP3 (F, M). iNKT were identified as positive for CD3 and Vα24Jα18-TCR (G, N).

The following monoclonal antibodies were used: αβTCR-FITC (FITC anti-human TCRα/β, clone IP26, BioLegend, USA); CD4-PE (anti-human CD4 PE, clone RPA-T4, BioLegend, USA); CD8-PerCP/Cy5.5 (anti-human CD8α PerCP/Cy5.5, clone RPA-T8, BioLegend, USA); FOXP3 PerCP/Cy5.5 (anti-human, clone PCH101, eBioscience, USA); CD3 PE-Cy5 (anti-human, clone UCHT1, eBioscience, USA); Vα24Jα18 TCR PE (anti-human clone 6B11, eBioscience, USA). Thymocytes have been distinguished from pDCs based on FSC/SSC parameters. One representative experiment out of nine independent experiments is shown.

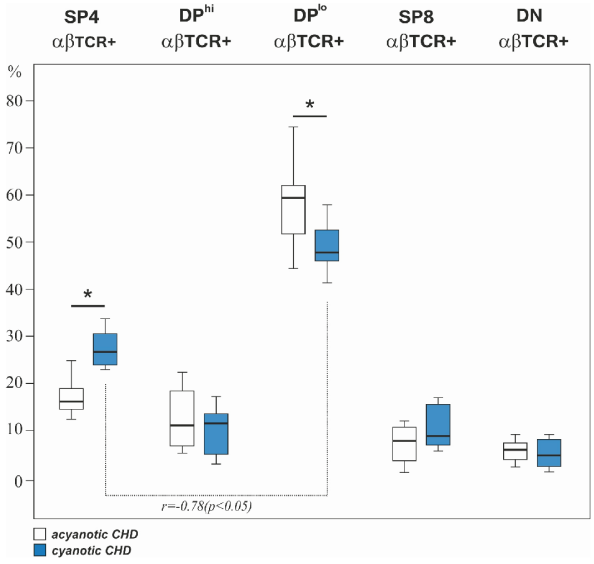

Figure 2. Distribution of the main CD4 and CD8 thymocyte subsets, expressing αβ chains of T-cell receptor (αβTCR$^+$ thymocyte) after 72h incubation with the plasmacytoid dendritic cells in cyanotic and acyanotic congenital heart diseases (CHD).

Percentage of DN (CD4$^-$CD8$^-$); DP with low expression of CD4 and CD8 (DP^{lo}) (CD4^{lo}CD8^{lo}); DP with high expression of CD4 and CD8 (DP^{hi}) (CD4^{hi}CD8^{hi}); SP4 (CD4^{hi}CD8$^{-/lo}$); SP8 (CD4$^{-/lo}$CD8^{hi}) in gated αβTCR$^+$ thymocytes are represented. Results are expressed as median with the lower and upper quartiles. Statistically significant differences between the groups of cyanotic and acyanotic CHD by t-test for independent samples (groups) are indicated (*; p=0.04) (n=9). The Spearman rank correlation coefficient (r) between the percentages of SP4 (CD4^{hi}CD8$^{-/lo}$) and DP^{lo} (CD4^{lo}CD8^{lo}) αβTCR$^+$ thymocytes in cyanotic CHD is presented.

The expressions of CD4 (anti-human CD4 PE, clone RPA-T4, BioLegend, USA) and CD8 (anti-human CD8α PerCP/Cy5.5, clone RPA-T8, BioLegend, USA) on thymocytes expressing T-cell receptor αβ-chain (αβTCR) (FITC anti-human TCRαβ, clone IP26, BioLegend, USA) were determined by flow cytometry.

Discussion

Here, we assessed the hypoxia influence in cyanotic CHD on main thymocyte subset distributions in the presence of thymic pDCs *in vitro*, covering not only the main stages of αβTCR cell differentiation but also nTreg and iNKT in comparison with acyanotic CHD. We used the reaggregated culture of unfractionated thymocytes co-cultivated with pDCs isolated from the same thymus of children with cyanotic or acyanotic (as a control) CHD *in vitro*. The reaggregated culture of unfractionated thymocytes with pDCs gives advantage to investigate the thymocyte development from undifferentiated precursors and the cell-to-cell effective interactions with pDCs (17,18). Although fetal thymus organ cultures model and another such as OP9-DL1 assays seems to provide the most natural conditions, it cannot be used to study the thymocyte development in CHD under hypoxia (17,18). A disruption of internal structure of reticular epithelial cells and a loss of their integrity with thymocytes was revealed in thymi of children depending on the severity of CHD and hypoxia (3,6,19). At the same time, immature pDCs accumulated in the thymus medulla and cortex of children with CHD (3,6,11). According to literature, pDCs due to major histocompatibility complex (MHC) I, MHC II, CD80/86 expressions and cytokine productions can mediate all the required signaling events for a positive or negative selection of thymocytes, covering nTreg and iNKT development (17,18). Therefore, pDCs may play an essential role in the positive and negative selection under thymus development disorders associated with CHD. The result of pDCs and thymocyte interactions depends on the avidity of TCR to

Table 1. The percentages of apoptotic cells and Bcl-2 expression in thymocytes after incubating for 72 h with the plasmacytoid dendritic cells (pDCs) in cyanotic and acyanotic congenital heart diseases (CHD).

	Cyanotic CHD	Acyanotic CHD	p
AnV$^+$/PI$^-$, % (early apoptosis)	50.6 (32.0-63.7)	49.1 (43.4-54.1)	
AnV$^+$/PI$^+$, % (late apoptosis)	20.1 (18.1-28.0)*	37.4 (36.5-53.1)	p=0.024
Bcl-2 expression in thymocytes in dexamethasone-induced apoptosis			
Bcl-2$^+$αβTCR$^+$, %	6.5 (5.9-7.1)*	2.7 (2.4-3.2)	p=0.004
Bcl-2$^+$CD4$^+$, %	5.2 (4.8-5.7)*	2.3 (1.0-3.0)	p=0.007
Bcl-2$^+$nTreg, %	12.8 (11.1-14.4)$^{\#}$	11.3 (11.3-15.5)$^{\#}$	
Bcl-2$^+$iNKT, %	20.0 (16.4-23.6)*	10.0 (6.6-10.3)	p=0.036
Bcl-2 expression in thymocytes			
Bcl-2$^+$αβTCR$^+$, %	5.4 (3.1-5.9)	3.3 (2.7-3.9)	
Bcl-2$^+$CD4$^+$, %	4.7 (2.8-4.9)	2.9 (2.3-3.6)	
Bcl-2$^+$nTreg, %	31.9 (22.7-32.7)	21 (20.6-21.3)	
Bcl-2$^+$iNKT, %	12.8 (5.3-13.6)	5.3 (4.5-6.1)	

The percentages of apoptotic cells: High Annexin V (AnV$^+$) and low (PI$^-$) or high (PI$^+$) Propidium Iodide (PI) staining; Bcl-2 expression in gate of thymocytes, expressing αβ chains of T-cell receptor (Bcl-2$^+$αβTCR$^+$); in gate of CD4$^+$ thymocytes (Bcl-2$^+$CD4$^+$); in gate of natural regulatory T-lymphocytes (Bcl-2$^+$nTreg); in gate of invariant natural killer T-cells (Bcl-2$^+$iNKT) are presented. Results are expressed as median with the lower and upper quartiles. Statistically significant differences between values in thymocytes in co-cultures with pDCs in cyanotic and acyanotic CHD by t-test for independent samples (groups) are indicated (*; p<0.05); statistically significant differences by t-test between Bcl-2 expression in thymocytes under influence of dexamethasone and without dexamethasone are indicated ($^{\#}$; p<0.05); n=9 in cyanotic CHD; n=9 in acyanotic CHD

peptide/MHC complex and secondary signals and provides either differentiation or apoptosis of thymocytes (18). In the present study, it was shown that thymocyte development during hypoxia in cyanotic CHD increased their resistance to apoptosis *in vitro*. This effect may be partially related to enhanced Bcl-2 expression in cyanotic CHD in comparison with acyanotic CHD, which was confirmed by previous immunohistochemical studies (3,6,20). Other authors have indicated that increased expression of HIF-1α under hypoxia in thymocytes regulates caspase activities (21) with little effect on Bcl-2 expression (22). We may propose that thymocyte resistance to apoptosis on TCR ligation is associated with enhancing Bcl-2 expression levels, but are unlikely to be the only determinant regulating susceptibility to apoptosis in the thymus (20,23,24). Other authors have appreciated that Bcl-2$^+$ thymocytes detected across the developmental stages of maturation undergo positive

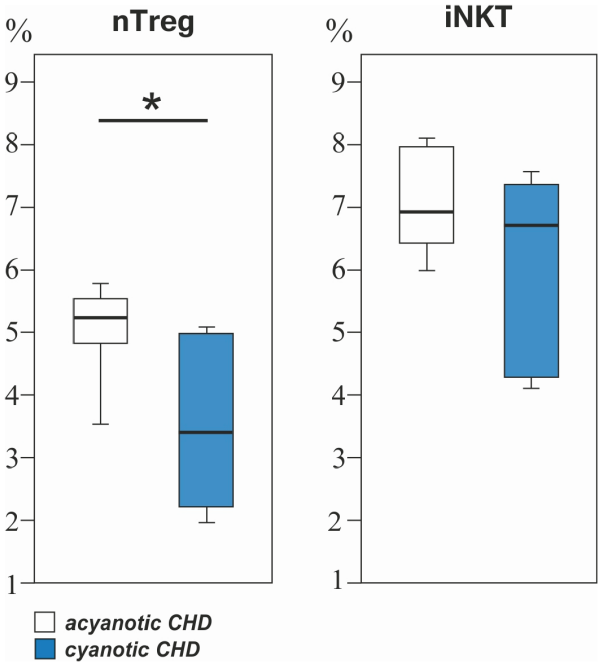

Figure 3. Natural regulatory T-lymphocytes (nTreg) precursor percentage **(A)** in the co-cultures of thymocytes after 72h cultivation with the plasmacytoid dendritic cells (pDCs) in cyanotic and acyanotic congenital heart diseases (CHD). The percentages of nTreg precursors (FOXP3$^+$cells in gated CD4$^+$CD25$^+$thymocytes) are represented. Results are expressed as median with the lower and upper quartiles. A statistically significant difference t-test for independent samples (groups) between cyanotic and acyanotic CHD (*; p=0.048) is indicated (n=9).
The following monoclonal antibodies were used: Human CD4$^+$(FITC) CD25$^+$(PE) regulatory T-cell staining reagent, R&D, USA; Anti-Human FOXP3 PerCP/Cy5.5, clone PCH101, eBioscience, USA.
(B) iNKT (invariant natural killer T-cells) precursor percentage in the co-cultures of thymocytes after 72h cultivation with pDCs in cyanotic and acyanotic CHD. The iNKT precursor percentages (CD3^{hi}Vα24Jα18-TCR$^+$thymocytes in gate of total thymocytes) are represented. Results are expressed as median with the lower and upper quartiles (n=9).
The following monoclonal antibodies were used: Anti-human CD3 PE-Cy5, clone UCHT1, eBioscience, USA; anti-human Vα24Jα18 TCR PE, clone 6B11, eBioscience, USA.

selection and survival under hypoxia-induced apoptosis (23-25). Prenatal hypoxia increased HIF-1α and caspase 3 expressions and decreased expression of the proliferation marker Ki-67 in the thymus in mice offspring (21). The authors propose that deficiencies in IL-2-mediated signaling are one of the sources of hypoxia-impaired thymocyte development (21). There were not differences in the proliferation marker Ki-76 expression in thymocyte populations between cyanotic and acyanotic CHD in our study. These data suggest that regulatory mechanisms exist to maintain thymic cellular under hypoxia in CHD *in vivo* and that hypoxia regulates thymocyte survival.

The incubation of thymocytes with pDCs *in vitro* stimulated CD4^{hi}CD8^{hi}αβTCR^{low} thymocytes to acquire CD4^{lo}CD8^{lo}αβTCR^{hi} phenotype, which is a characteristic of positive selection regardless of hypoxia, and at least a fraction did so without undergoing apoptosis, confirming former works (17,18). Co-localization of DP thymocytes with pDCs in thymus cortex in children with CHD assumed the ability of pDCs to participate in thymocyte positive selection in CHD *in vivo*.

The thymocyte development during hypoxia in cyanotic CHD resulted in a decreased number of CD4^{lo}CD8^{lo}αβTCR$^+$ thymocytes in co-cultures with pDCs, that may be due to their transformation into CD4^{hi}CD8$^{-/lo}$αβTCR$^+$ cells, without affecting the formation of CD4$^{-/lo}$CD8^{hi}αβTCR$^+$ thymocytes *in vitro*. According to the literature, the hypoxia in cyanotic CHD stimulates the hypoxia-inducible factor - 1α (HIF-1α) expression (20) that changes the expression of genes controlling the CD4 and CD8 molecule expressions (22). It should be noted that obtained data do not exclude the possibility of that some parts of the CD4^{lo}CD8^{lo}αβTCR$^+$ cells undergo to apoptosis. The presented results may help explaining the reduction in absolute T-lymphocyte number, as well as the altered ratio of CD4 to CD8 T-lymphocytes in the peripheral blood of children with CHD, which is mostly due to CD4 lymphocyte changes because CD8 T-lymphocyte numbers do not change (26,27).

nTreg cells develop in the thymus from DP αβTCR$^+$ thymocytes resistant to apoptosis upon contact with antigen-presenting cells, including pDCs of the medulla during negative selection (28). Histologic evidence was provided that pDC-nTreg co-localization within the human thymic medulla, which may also support a functional interaction between the 2 cell types *in vivo* (10). TCR recognition of the peptide/MHC class II complexes on the thymic DCs and the signal from the CD28 and CD80/86 interaction induce the expression of CD25 molecules and FOXP3 transcription factor in nTreg precursors (28). The thymocytes and pDCs development under hypoxia in CHD decreased CD4$^+$CD25$^+$FOXP3$^+$ cells in their co-cultures *in vitro* in comparison with acyanotic CHD. It may be explained by

the accumulation of immature pDCs in thymic medulla under hypoxia in CHD. We have proposed that hypoxia in cyanotic CHD influenced pDCs' functional activity. Therefore, the decreased number of nTreg precursors in thymocyte co-cultures with pDCs in cyanotic CHD may be related to lower IL-10 production with higher IL-12 (p40) synthesis by pDCs. Moreover, HIF-1α transcription factor was over-expressed in thymocytes during hypoxia which is an antagonist for the FOXP3 expression (20,29,30). In our study, nTreg precursors differed from other thymocytes by upregulated Bcl-2 expression that decreased by dexamethasone influence that was in line with other studies (31). There were no differences in Bcl-2 expression in nTreg precursors between cyanotic and acyanotic CHD, which may be connected with high level of background Bcl-2 expression (31). As the main function of nTreg is to control autoimmune processes, our results might help explaining the reduction in Treg numbers in peripheral blood, as well as the high frequency of allergic and autoimmune pathologies of children with cyanotic CHD (26).

iNKT develops in the thymus from DP $\alpha\beta TCR^{+}$ thymocytes during positive selection (28). iNKT is selected by lipid antigens presented by the non-polymorphic, MHC I-like molecule CD1d, existing on the surface of other DP $\alpha\beta TCR^{+}$ thymocytes, and requires additional signals from the signalling lymphocytic-activation molecule family of receptors and Notch-signaling (28). CD1d-expressing thymic DCs mediate negative selection of iNKT, followed by their proliferation and differentiation into different iNKT subpopulations (28). We detected that thymic pDCs were involved in the induction of the $CD3^{hi}V\alpha24J\alpha18^{+}$ cell formation *in vitro*. Thymocyte development during hypoxia in cyanotic CHD did not affect the $CD3^{hi}V\alpha24J\alpha18^{+}$ cell formation in the presence of thymic pDCs but enhanced Bcl-2 expression in iNKT precursors *in vitro*. Decrease of iNKT cells in number in hypoxia in CHD have never been studied. Only a few studies have shown decrease of iNKT cells in peripheral blood of children with CHD in combination with Down syndrome (26). Considering that iNKT cells plays a leading role in immune response initiation due to rapid and massive cytokine production, changes in number of iNKT cells in CHD with hypoxia are caused by severe infection-related complications as myocarditis and endocarditis in CHD (7,19).

Conclusion

In the present study, we have demonstrated that thymocyte development during hypoxia in cyanotic CHD affected the thymocyte resistance to apoptosis, the differentiation of $CD4^{lo}CD8^{lo}\alpha\beta TCR^{+}$ thymocytes into $CD4^{hi}CD8^{-/lo}\alpha\beta TCR^{+}$ cells, the formation of nTreg precursors in thymocyte co-cultures with pDCs *in vitro*. The obtained results are important for understanding the underlying mechanism of immune dysfunctions in CHD and for improving the patient treatment.

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Ethics

Ethics Committee Approval: This study was approved by the local Ethics Committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences in accordance with the Declaration of Helsinki.

Informed Consent: Written informed consents were obtained from the patients' parents.

Peer-review: Internally and externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: N.L., R.S., Concept: E.O., N.L., S.S., Design: E.O., O.L., N.L., S.S., Data Collection or Processing: O.L., Analysis or Interpretation: E.O., O.L., R.S., S.S., Literature Search: E.O., O.L., R.S., S.S., Writing: E.O., N.L., R.S., S.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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Supplement Table 1. The percentages of αβTCR$^+$ thymocytes (expressing αβ chains of T-cell receptor) in gate of freshly isolated thymocytes (R1) and in gate of thymocytes after 72h co-cultivation with plasmacytoid dendritic cells (R2) in group of cyanotic and acyanotic congenital heart diseases (CHD).

αβTCR$^+$ thymocytes in freshly isolated thymocytes (R1), %		**αβTCR$^+$ thymocytes in R2, %**	
Cyanotic CHD	**Acyanotic CHD**	**Cyanotic CHD**	**Acyanotic CHD**
1	2	3	4
33.6 (22.7-45.8)	35.8 (31.2-47.1) P^{1-2}=0.07	58.0 (52.7-71.5) * P^{1-3}=0.048	68.8 (55.0-70.3) * P^{2-4}=0.049 P^{3-4}=0.06

CHD: Congenital heart diseases, Results are expressed as median with the lower and upper quartiles (n=7). Statistically significant differences by t-test for independent samples (groups) are indicated (*; p<0.05)

Supplement Table 2. The percentages of Ki-67$^+$ cells in different thymocyte subsets.

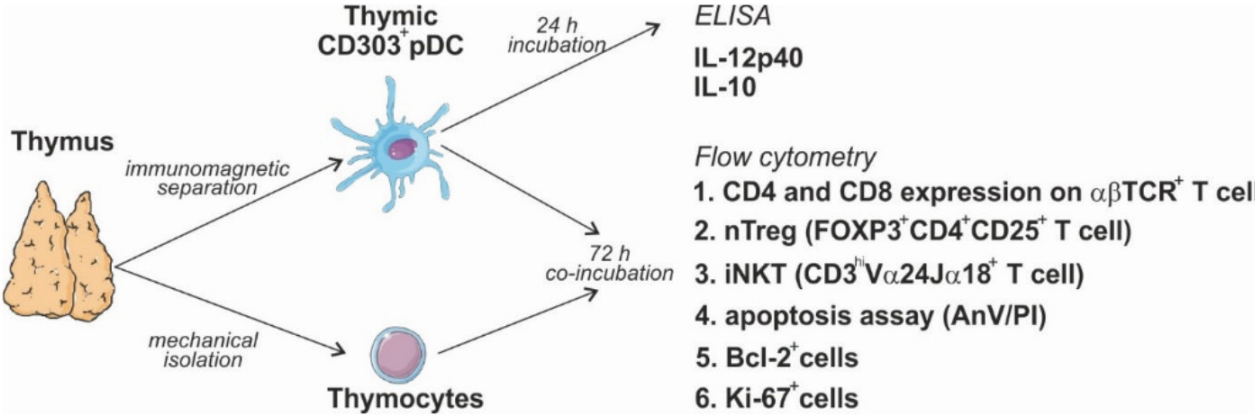
	Ki-67$^+$αβTCR+, %	**Ki-67$^+$CD4+, %**	**Ki-67$^+$nTreg, %**	**Ki-67$^+$iNKT**
Cyanotic CHD (n=2)	4.7 (3.3-6.2)	2.9 (2.6-3.3)	26.8 (23.8-29.9)	6.5 (4.0-9.1)
Acyanotic CHD (n=4)	2.5 (1.6-3.8)	2.4 (1.6-4.3)	30.7 (23.9-37.1)	14.8 (8.5-18.5)

CHD: Congenital heart diseases, iNKT: Invariant natural killer T-cells, nTreg: Natural regulatory T-cells, results are expressed as median with the lower and upper quartiles. The percentages of Ki-67$^+$ cells in gate of thymocytes, expressing αβ chains of T-cell receptor (Ki-67$^+$αβTCR$^+$); in gate of CD4$^+$ thymocytes (Ki-67$^+$CD4$^+$); in gate of natural regulatory T-lymphocytes (Ki-67$^+$nTreg); in gate of invariant natural killer T-cells (Ki-67$^+$iNKT) in the co-cultures with plasmacytoid dendritic cells after 72h incubation in groups of cyanotic and acyanotic CHD are presented. There were not statistically significant differences between groups of cyanotic and acyanotic CHD by t-test for independent samples (groups)

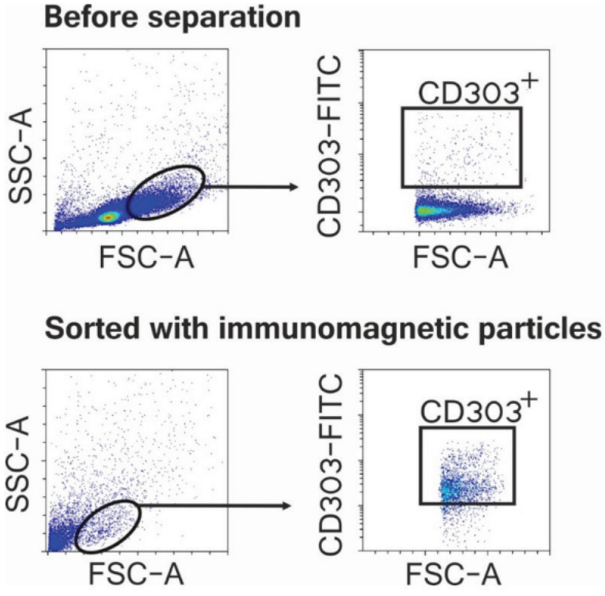
Supplement Table 3. The percentages of iNKT (invariant natural killer T-cells) and nTreg (natural regulatory T-lymphocytes) precursors in thymocyte cultures alone and with plasmacytoid dendritic cells (pDCs) in the groups of cyanotic and acyanotic congenital heart diseases (CHD).

		In acyanotic CHD		**In cyanotic CHD**	
		iNKT, %	**nTreg, %**	**iNKT, %**	**nTreg, %**
1	In freshly isolated thymocytes	1.76 (1.49-2.29)	0.37 (0.21-0.5)	1.59 (1.35-2.09)	0.29 (0.22-0.45)
2	After 72h thymocyte incubation alone	4.67 (1.79-8.45)	0.46 (0.36-0.8)	5.15 (1.41-7.81)	0.44 (0.29-0.75)
3	After 72h incubation with pDCs	6.93 (6.44-7.92)* p^{1-3}=0.005 p^{2-3}=0.006	5.24 (4.88-5.64)* p^{1-3}=0.02 p^{2-3}=0.001	6.78 (4.24-7.32)* p^{1-3}=0.009 p^{2-3}=0.009	3.28 (2.22-4.91)*# p^{1-3}=0.03 p^{2-3}=0.04 $p^{\#}$=0.048

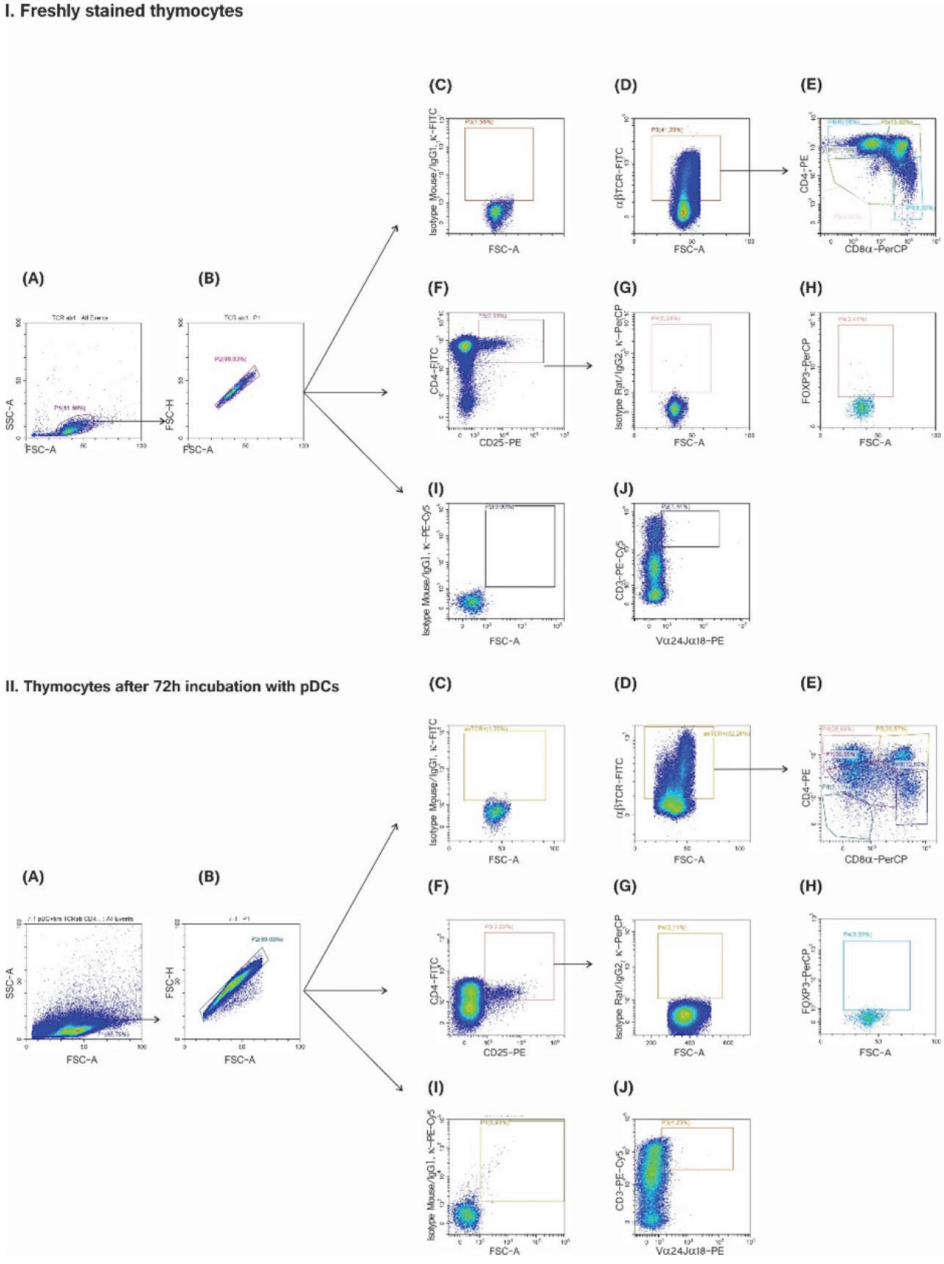
CHD: Congenital heart diseases, iNKT: Invariant natural killer T-cells, nTreg: Natural regulatory T-lymphocytes, results are expressed as median with the lower and upper quartiles (n=9). Statistically significant differences by t-test for independent samples (groups) are indicated (*; p<0.05). Statistically significant differences by t-test between corresponding parameters in cyanotic and acyanotic CHD are indicated (#; p<0.05).



Supplement Figure 1A. Design of experiments. Thymus fragments of children under 1 year of age with cyanotic or acyanotic congenital heart diseases (CHD) were cut into small pieces and thymocytes were released by mechanical pressing the tissue lightly. Plasmacytoid dendritic cells (pDCs) were sorted from the same thymus using immunomagnetic isolation kit. Purified pDCs were mixed with autologous thymocytes in ratio 1:10 (1×10^5:1×10^6 cells/mL) and incubated together for 72h. After incubation the aggregates were harvested, washed and stained with monoclonal antibodies. Supernatants were collected from pDC cultures alone. The levels of interleukin (IL) -10 and IL-12 (p40) in the pDCs culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Phenotype analysis were performed by flow cytometry for evaluation of different thymocyte subsets, expressing αβ chains of T-cell receptor (αβTCR), based on CD4 and CD8 expression, and also invariant natural killer T-cells (iNKT), natural regulatory T-lymphocytes (nTreg). Thymocytes were additionally stained with Annexin V and Propidium Iodide (PI) for apoptosis assay and stained for intracellular Ki-67 and Bcl-2 expression determination in different thymocyte subpopulations.



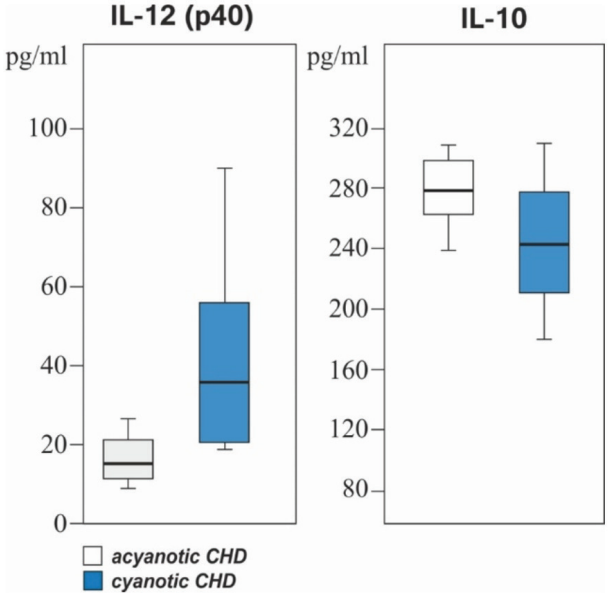
Supplement Figure 1B. Flow cytometry analysis of plasmacytoid dendritic cells (pDCs) ($CD303^+$) before separation and after purified by immunomagnetic separation from human thymus fragments. Shown are representative plots of DCs based on forward versus side scatter (FSC/SSC) and further gated with basis on the expression of CD303 for pDCs. The purity of the isolated pDCs verifying by flow cytometry with monoclonal antibodies to CD303 [anti-human CD303 (BDCA-2) FITC, clone 201A, BioLegend, USA]. One representative experiment out of nine independent experiments is shown.



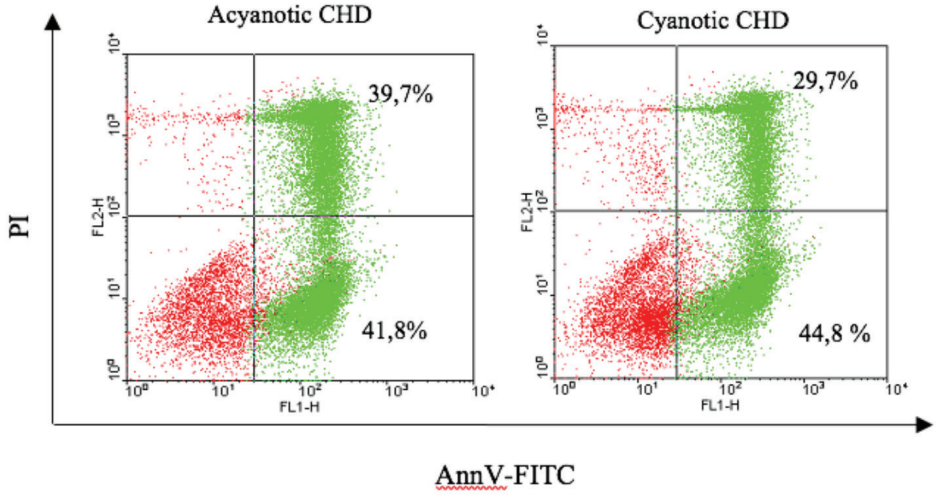
Supplement Figure 2. Flow cytometry analysis of freshly unfractionated thymocytes isolated from thymus (I) and unfractionated thymocytes co-cultured for 72h with the plasmacytoid dendritic cells (pDCs) (II). The gating strategy depicted here was used for analyzing the main thymocyte subsets, expressing αβ chains of T-cell receptor (αβTCR thymocyte), natural regulatory T-lymphocytes (nTreg), invariant natural killer T-cells (iNKT) in unfractionated thymocyte (R1) and after 72h co-cultivation with pDCs (R2).

Representative dot plots showed the gate used to identify thymocytes (R1) for freshly isolated thymocytes (A); R2 for thymocytes after co-cultivation for 72h with the pDCs (H) based on forward versus side scatter (FSC/SSC); next step for identification of viable cells and gated out of doublets and dead cells based on FSC-A/FSC-H (B, I); αβTCR$^+$ thymocytes were identified by αβTCR expression (D) and the different thymocyte subsets were further gated (E) based on the expression of CD4 and CD8 markers (CD4$^-$CD8$^-$; CD4^{lo}CD8^{lo}; CD4^{hi}CD8^{hi}; CD4^{hi}CD8$^{-/lo}$; CD4$^{-/lo}$CD8^{hi}). nTreg were defined within CD4$^+$CD25$^+$ thymocytes (F) and expressing FOXP3 (H). iNKT were identified as positive for CD3 and Vα24Jα18-TCR (J).

The following monoclonal antibodies were used: αβTCR-FITC (FITC anti-human TCRα/β, clone IP26, BioLegend, USA); CD4-PE (anti-human CD4 PE, clone RPA-T4, BioLegend, USA); CD8-PerCP/Cy5.5 (anti-human CD8α PerCP/Cy5.5, clone RPA-T8, BioLegend, USA); FOXP3 PerCP/Cy5.5(anti-human, clone PCH101, eBioscience, USA); CD3 PE-Cy5 (anti-human, clone UCHT1, eBioscience, USA); Vα24Jα18 TCR PE (anti-human clone 6B11, eBioscience, USA). Appropriate isotype-matched control antibodies were included: for analysis of αβTCR$^+$ identification (FITC Mouse IgG1, κ, Isotype Ctrl Antybody clone MOPC-21) (C); for analysis of FOXP3$^+$staining (Rat IgG2a k Isotype Control (eBR2a), PerCP-Cyanine5.5, eBioscience, USA) (G); for analysis of CD3 PE-Cy5-and Vα24Jα18 TCR-PE staining (PE-Cy™5 Mouse IgG1 κ Isotype ControlClone MOPC-21 (RUO); Mouse IgG1 kappa Isotype Control (P3.6.2.8.1), PE, eBioscience™) (I). Flow cytometry was performed on a CytoFlexS using CytExpert 2.4 software (Beckman Coulter, USA). One representative experiment out of nine independent experiments is shown. Thymocytes have been distinguished from pDCs basing on FSC/SSC parameters.



Supplement Figure 3. Cytokine productions by thymic plasmacytoid dendritic cells (pDCs) in cyanotic and acyanotic congenital heart diseases (CHD). Interleukin (IL) -10 and IL-12 (p40) concentrations (pg/mL) were detected in the supernatants of thymic pDCs. Results are expressed as median with the lower and upper quartiles. There were independent experiments: in cyanotic (n=7) and acyanotic (n=7) CHD.



Supplement Figure 4. Apoptosis assessed by Annexin V/Propidium Iodide (PI) assay. Thymocytes of children with cyanotic and acyanotic congenital heart diseases (CHD) after incubating with thymic plasmacytoid dendritic cells for 72 hours were stained with Annexin V-FITC and PI and analyzed by flow cytometry. The results are presented as density plots of PI vs. Annexin V-FITC. Apoptotic cells have high Annexin V-FITC and low or high PI staining (lower-right and up-right quadrants). Thymocytes consistently bound high levels of Annexin V, a ligand for phosphatidylserine and a marker of early apoptosis. Thymocytes also bound low levels of PI, a nucleic acid marker consistent with late apoptosis. Numbers on the graph represent the percentages of apoptotic cells in the different groups from one representative experiment. Data shown are from a single experiment representative of nine performed.