Fetal and Postnatal Development of T-lymphocyte Subpopulations

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Abstract

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Changes in individual subpopulations of lymphocytes (mainly $CD2^+$, $CD4^+$ and $CD8^+$) in primary and secondary lymphatic organs and circulating blood were observed in pig fetuses between days 51 and 112 of gestation, and in circulating blood in postnatal piglets. The technique of flow cytofluorimetry was used and binding of specific monoclonal antibodies was visualised using polyclonal antibodies against mouse or rat Ig marked with fluorochroms (PE and FITC). As soon as on day 51 of gestation, $CD4^+$ and $CD8^+$ T lymphocytes were demonstrated in porcine thymus. Their relative and actual numbers continued to increase markedly. When this increase was expressed in terms of age with subsequent intrapolation, the changes in $CD4^+/CD8^+$ phenotype expression could be expected around day 40, i.e. in the period, when thymus cortex and medulla are not yet morphologically differentiated.

In the spleen only $CD2^+$ cells were found on day 51 of gestation. Expression of lymphocytes with $CD4^+$ and $CD8^+$ receptors was shown on day 60. Their relative and actual numbers increased with age. This increase when expressed per whole organ made a difference of three orders of magnitude. In lymph nodes, only changes from day 90 were followed. In this secondary lymphatic organ, the percentage of T lymphocytes with $CD4^+$ and $CD8^+$ markers was higher than that in the spleen. The $CD4^+/CD8^+$ ratio in spleen and thymus gradually decreased with advancing age to 1 with a slightly dominant $CD4^+$ lymphocyte subpopulation. On the other hand, in lymph nodes of pig fetuses $CD8^+$ lymphocytes prevailed (index 0.85). In the postnatal period, a marked increase of cytotoxic $CD8^+$ lymphocytes occurred in peripheral blood of 28-day-old piglets. Thus the $CD4^+/CD8^+$ index decreased from 1 to 0.2. This characteristic of lymphocyte subpopulations in circulating blood is also typical of adult individuals. The numbers of B lymphocytes with IgM receptors in circulating blood increased gradually from day 90 of prenatal development until day 28^h of postnatal life both in relative and actual terms.

Prenatal ontogenesis, pig fetuses, lymphatic organs, quantitative cytology

Ontogenetic development of the individual lymphocyte subpopulations in lymphatic and haemopoietic organs has been in focus of interest of many research laboratories. As suitable experimental models for these studies animal species are used in which placentation preventing the transport of immunoglobulins between mother and fetus, i.e. those equipped with epitheliochorial or syndesmochorial placenta (Šterzl and Silverstein 1967). Therefore we used the porcine experimental model with an epitheliochorial type of placenta that fulfils the above-mentioned criteria (Plate III and IV, Fig. 1 and 2). Moreover, this model offers some other advantages such as appropriate litter size for the statistical analysis, a sufficiently long period of gestation (114 days) so that the individual developmental periods do not overlap, relatively good accessibility of this model and previously elaborated anaesthetic and surgical techniques in pregnant animals (Kovářů et al. 1971; Kovářů and Stožický 1986; Řeháková et al. 1996).

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Various aspects of the development of lymphatic and haemopoietic organs in this animal species such as quantitative morphology, quantitative cytology, cell biochemistry and molecular biology have been described in detail (Kovářů et al. 1979; Kovářů et al. 1987; Kovářů et al. 1988ab; Kovářů et al. 1994; Kovářů et al. 1995).

The first T lymphocytes have been documented in the epithelial base of thymus as soon as on day 28 (FD) of fetal development using polyclonal sheep anti–pig T cell antiserum with CD2 specificity (Trebichavský et al. 1985). The first B lymphocytes with IgM receptor were described in the liver on FD 44 (Prokešová et al. 1979 ab). Thus it is possible to speak about T – B dichotomy during the second month of prenatal development (Kovářů et al. 1978) and it has been demonstrated that each lymphocyte is bearing only T or B phenotype (Jarošková and Kovářů 1978). This was done using two types of labelling (production of spontaneous E-rosette forming cells as T markers and demonstration of IgM receptor as a B cell marker by microautoradiography.

Therefore the aim of this study is to further characterize the individual subpopulations of mainly T lymphocytes, i.e. to perform a qualitative and quantitative analysis of helper (CD4) and cytotoxic (CD8) T lymphocyte subpopulation as well as CD2 cells during the prenatal and early postnatal pig life in primary and secondary lymphatic organs, and in circulating blood. Cytological changes were expressed as percentage of total amount, related to one gram of wet weight or to whole organ mass, suggesting the capacity of immune system in respect to different effector structure elements, because there are no quantitative data in published papers dealing with these problems.

Materials and Methods

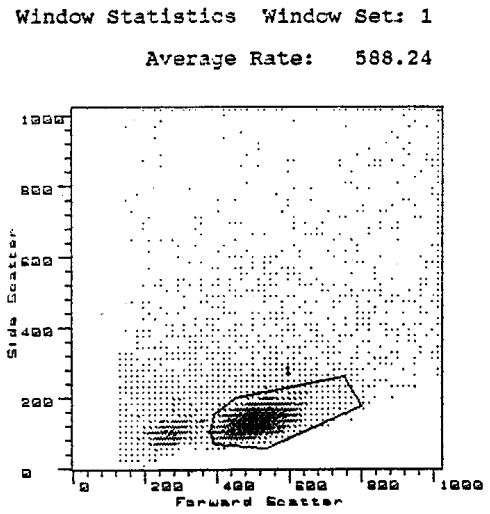
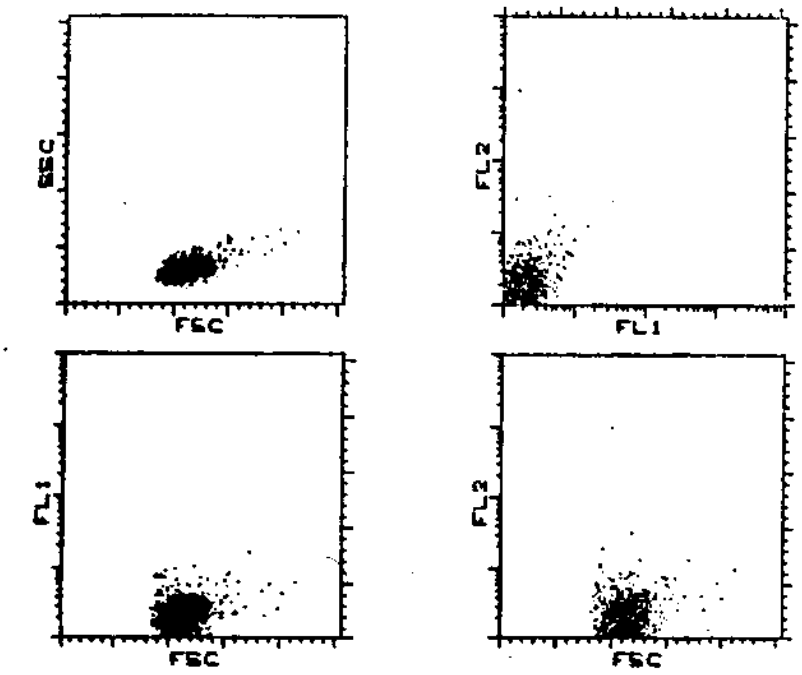


Fig. 3. Typical working "window" for pig lymphocytes in measured cell suspensions characterized relative values of side and forward scatter

Fetuses of 5 pregnant sows (Czech LargeWhite) from FD 55 to FD 112 (a total of 34 fetuses) were used. Further, blood samples from 8 piglets of the same breed were collected on postnatal day (PD) 28. For obtaining fetuses during the prenatal development the technique for fetal surgery and halothan-O_2 anaesthesia of the mother with maintenance of basal physiological indices in fetuses and mother (Kovářů et al. 1971; Kovářů and Stožický 1986; Řeháková et al. 1996). For isolation of the cells from the primary and secondary lymphatic organs and their characterization the techniques of classical and quantitative cytology were used (Kovářů et al. 1987; Kovářů et al. 1992) with the following separation of lymphocyte subpopulations on discontinous gradient of Histopaque 1077 (Sigma). For phenotyping of the individual lymphocyte populations murine monoclonal antibodies (MoAb) against pig T lymphocytes PT4 (anti-CD4) and SL2 (anti-CD8) and rat MoAb against pig CD2 receptor (Mac80) were used. CD4 and CD8 MoAbs were provided by Dr. Rothkotter from the University of Medical Sciences, Hannover. MoAbs were developed at the University of Tübingen (Germany) and CD2 MoAb was kindly provided by Dr. Binns, Babraham Institute of Animal Physiology, Cambridge (United Kingdom). Mouse MoAbs against IgM receptor (Lig4) used only for lymphocytes of circulating blood originated from Ing. Petr Dvořák's laboratory, Academy of Sciences of the Czech Republic. As 2nd antibodies we used phycoerythrin-Fab fragment conjugate of goat anti-mouse polyclonal Ig (Becton Dickinson) as well as conjugate of FITC-rabbit anti-mouse (or rat) polyclonal Ig (DaKo immunoglobulins). Cells were analysed by flow cytofluorimetry (FACS) analysis (Becton Dickinson). Typical histograms of the individual subpopulation of T lymphocytes, including working "window" for pig lymphocytes characterized relative levels side and forward scatter and set up of the background using controls are depicted in Figs 3, 4 and 5.



CONTROL - THYMUS ONLY

PE CONJUGATE

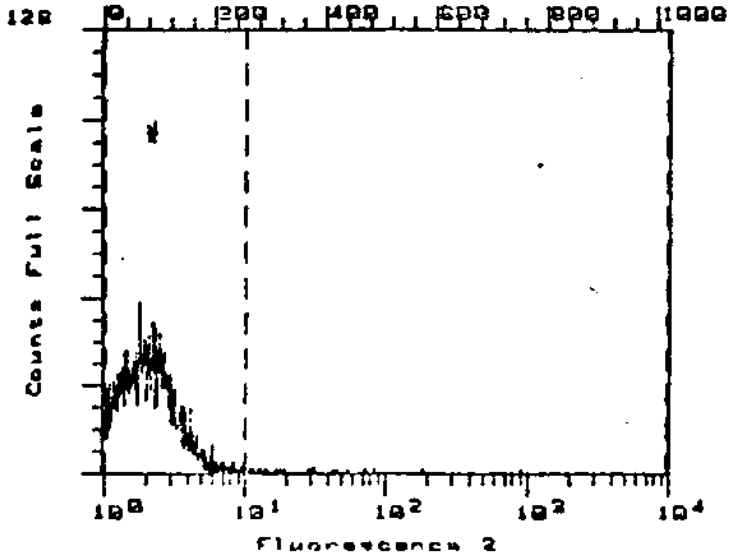


Fig. 4. Histogram of control activity of thymus cells with PE conjugate

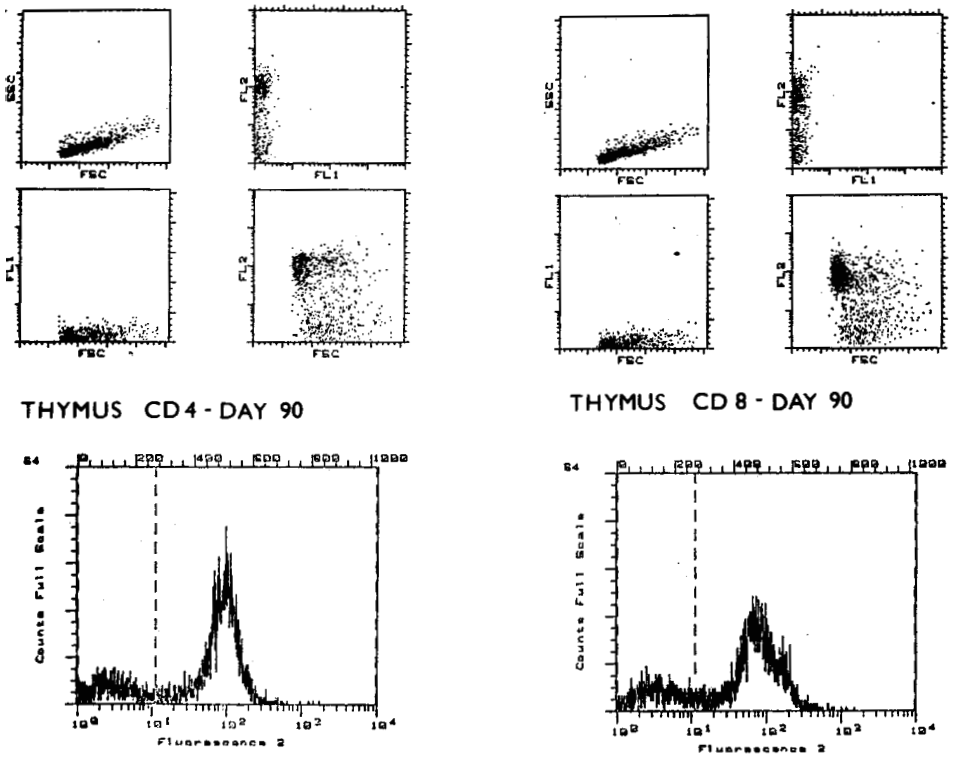


Fig. 5. Histogram of $CD4^+$ and $CD8^+$ thymic lymphocytes

Results and Discussion

In the thymus, a primary lymphatic organ, $CD2^+$ and also $CD4^+$ and $CD8^+$ T lymphocytes were confirmed as soon as on FD 51. Their percentage proportion rose significantly from FD 51 to FD 60 ($CD2^+$ and $CD4^+$). For $CD8^+$ T lymphocytes this increase was evident until FD 90 (Table 1). When expressed per 1 gram of wet weight and per whole organ mass for all three categories of thymus lymphocytes, this increase is evident until FD 90 and FD 112,

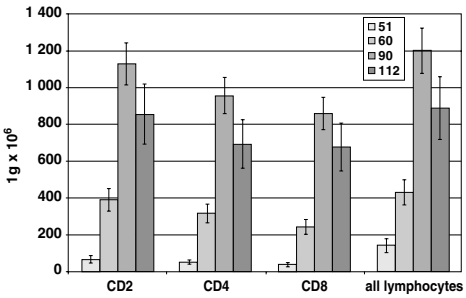


Fig. 6. Count of T-lymphocytes with $CD2^+$, $CD4^+$ and $CD8^+$ receptors in thymus during prenatal development (per 1 gram w.w.)

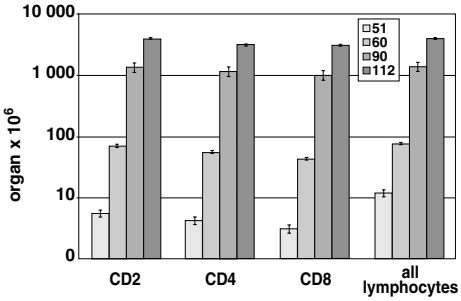


Fig. 7. Count of T-lymphocytes with $CD2^+$, $CD4^+$ and $CD8^+$ receptors in thymus during prenatal development (per whole organ)

Table 1
Proportion of T-lymphocytes with $CD2^+$, $CD4^+$ and $CD8^+$ receptors during prenatal development (%)

Gestation (days)		51	n	60	n	90	n	112	n
Thymus	$CD2^+$	45.5 ± 3.1	7	90.0 ± 7.8	7	93.5 ± 6.5	13	96.1 ± 7.5	7
	$CD4^+$	35.1 ± 2.9	7	73.5 ± 3.1	7	79.2 ± 4.1	13	77.7 ± 5.2	7
	$CD8^+$	26.0 ± 2.1	7	57.0 ± 2.7	7	71.3 ± 3.2	13	76.2 ± 4.9	7
	$CD4^+/CD8^+$	1.35 ± 0.24	7	1.29 ± 0.12	7	1.11 ± 0.11	13	1.02 ± 0.14	7
spleen	$CD2^+$	0.2 ± 001	7	4.4 ± 0.2	7	12.9 ± 0.8	13	45.2 ± 2.1	7
	$CD4^+$	< 0.01	7	3.0 ± 0.3	7	5.6 ± 0.3	13	33.7 ± 1.8	7
	$CD8^+$	< 0.01	7	0.7 ± 0.1	7	3.8 ± 0.2	13	20.0 ± 1.2	7
	$CD4^+/CD8^+$	ND	7	4.29 ± 1.21	7	1.47 ± 0.17	13	1.65 ± 0.24	7
lymph nodes	$CD2^+$	ND	7	ND	7	75.1 ± 4.3	13	84.1 ± 6.5	7
	$CD4^+$	ND	7	ND	7	51.3 ± 3.1	13	57.2 ± 7.2	7
	$CD8^+$	ND	7	ND	7	60.0 ± 3.7	13	67.5 ± 3.9	7
	$CD4^+/CD8^+$	ND	7	ND	7	0.6 ± 0.11	13	0.85 ± 0.12	7

respectively, and it represents a difference of two and three orders of magnitude, respectively (Fig. 6 and 7). When the curve of subpopulation changes expressed as a function of age was interpolated, it could be suggested that $CD4^+/CD8^+$ phenotype was present around FD 40, i.e. in the period, when the cortex and medulla in thymus were not yet morphologically differentiated (Kruml et al. 1970 ab). $CD4^+/CD8^+$ ration in this organ decreases from the value 1.35 on FD 51 to 1.02 on FD 112 (Table 1). In the spleen, the secondary lymphatic

Table 2
Proportion of lymphocytes with $CD2^+$, $CD4^+$, $CD8^+$ and $sIgM^+$ receptors in circulating blood during prenatal and early postnatal ontogenesis

Gestation (days)	FD90	n	FD112	n	PD28	n
CD2	61.50 ± 3.50	9	68.30 ± 5.20	7	80.40 ± 2.00	8
CD4	11.80 ± 1.10	9	10.90 ± 1.80	7	11.40 ± 0.90	8
CD8	13.60 ± 3.00	9	18.20 ± 2.80	7	54.00 ± 4.20	8
CD4 / CD8	0.87 ± 0.10	9	0.60 ± 0.05	7	0.21 ± 0.01	8
S IgM	9.20 ± 2.50	9	11.80 ± 1.90	7	16.50 ± 2.80	8
CD2 / S IgM	6.68 ± 1.45	9	5.79 ± 0.42	7	4.87 ± 0.74	8

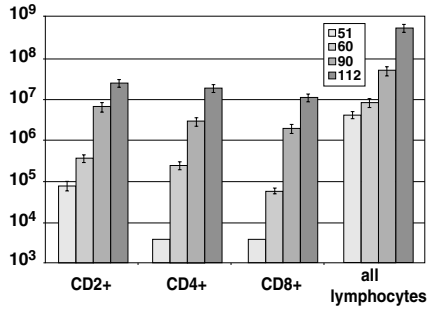


Fig. 8. Count of T-lymphocytes with $CD2^+$, $CD4^+$ and $CD8^+$ receptors in spleen during prenatal development (per 1 gram w.w.)

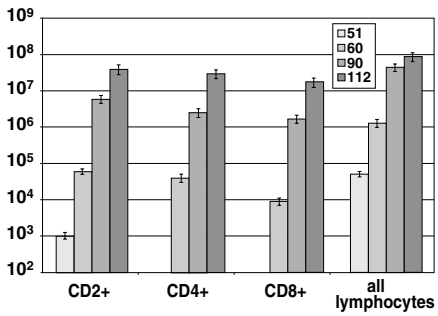


Fig. 9. Count of T-lymphocytes with $CD2^+$, $CD4^+$ and $CD8^+$ receptors in spleen during prenatal development (per whole organ)

Table 3
Counts of T-lymphocytes with $CD2^+$, $CD4^+$ and $CD8^+$ receptors in mesenteric lymph nodes during prenatal development (10^6)

	per 1 gram w. w.		per whole organ	
Gestation (days)	90	112	90	112
$CD2^+$	4.30 ± 0.71	4.75 ± 1.19	50.62 ± 8.86	126.64 ± 26.16
$CD4^+$	2.94 ± 0.49	3.23 ± 0.76	34.58 ± 6.05	84.77 ± 17.79
$CD8^+$	3.44 ± 0.54	3.81 ± 0.90	40.44 ± 7.08	100.04 ± 20.99
all lymphocytes	5.73 ± 0.95	5.65 ± 1.33	67.40 ± 11.80	148.20 ± 31.10

organ, only lymphocytes expressing $CD2^+$ marker on FD 51 were confirmed. We assume that it was the T lymphocyte population, although low expression of this phenotype was also confirmed for B cells in pigs (Šinkora et al. 1998b, 2000). Expression of the surface markers of helper ($CD4^+$) and cytotoxic ($CD8^+$) lymphocytes was not confirmed and was apparently below the level of detection. On FD 60 both of them and also CD^{2+} of all $CD2^+$ followed by their significant increase up to FD 112 (Table 1). When expressed per 1 gram of wet weight of the spleen and to the whole organ mass this increase represented as a function of age, a difference of two or three orders of magnitude, respectively (Fig. 8 and 9). $CD4^+/CD8^+$ ratio decreased in the spleen between FD 60 and FD 112 significantly from 4.29 to 1.65 (Table 1). This was caused mainly by an increased number of cytotoxic $CD8^+$ lymphocytes. Expression of IgM receptors on the surface of the spleen lymphocytes was not carried out in these experiments. Changes in T lymphocyte subpopulations were estimated only in mesenterial lymph nodes on FD 90 to FD 112 due to technical reasons (Table 1 and 3). Percentage proportion of the individual subpopulations of T lymphocytes was in this secondary lymphatic organ significantly higher than in spleen (Table 1). However, when expressed per 1 gram of wet weight this difference disappeared (Table 3) due to low cellularity of lymph nodes, because of loosely diffused lymphocytes in their histioreticular structure. When related to the whole organ, this trend was more apparent again, since total weight of the mesenterial lymph nodes was significantly higher (Table 3). $CD4^+/CD8^+$ ratio decreased in this organ below value 1, which is given by $CD8^+$ subpopulation mainly in comparison to other lymphatic organs. In circulating blood we observed expression of the individual subpopulations of T and B lymphocytes with membrane IgM receptor from FD 90 to PD 28. It is evident from Table 2 that postnatally the percent proportion of $CD8^+$ lymphocytes increased significantly, apparently due to antigenic pressure of microbial flora. This is particularly evident in relation to 1 L of circulating blood, where this increase reached a value of one order of magnitude higher (Fig. 10). $CD4^+/CD8^+$ index decreased in this case from 0.87 to 0.21. This characteristic of lymphocyte subpopulations is typical of circulating blood of adult pigs (Boeker et al. 1999; Saalmuller et al. 2001). The numbers of B lymphocytes with IgM receptor in circulating blood gradually increased during the whole observed period so that on PD 28 of postnatal life they approached values very similar to those of adult pigs, both in relative and actual terms (Table 2, Fig. 10). When $CD4^+$ and $CD8^+$ lymphocytes proportions are taken together and compared to $CD2^+$ cells, it is evident that

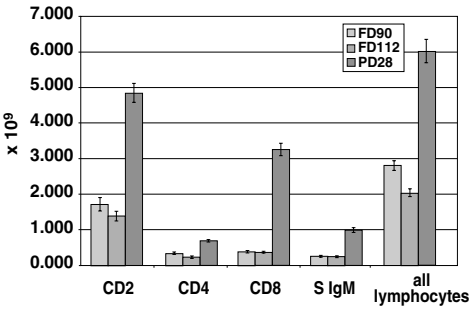


Fig. 10. Proportion of lymphocytes with $CD2^+$, $CD4^+$, $CD8^+$ and sIgM receptors per one litre of circulating blood during prenatal and early postnatal ontogenesis

in thymus, lymph nodes and spleen beginning on FD 51, FD 90 and FD 112, respectively, T lymphocytes expressed both $CD4^+$ and $CD8^+$ phenotypes. This is confirmed in other papers, where techniques of double and triple marking were used (Šinkora et al. 1998a; Trebichavský et al. 1995; Zuckermann et al. 1999.

Vývoj subpopulací T lymfocytů během prenatálního a postnatálního období života

U prasečích fétů od 51. do 112. dne gestace a v cirkulující krvi rovněž u postnatálních selat jsme sledovali změny v zastoupení jednotlivých subpopulací lymfocytů (především $CD2^+$, $CD4^+$ a $CD8^+$) v primárních a sekundárních lymfatických orgánech a cirkulující krvi. Pro tato sledování bylo použito techniky průtokové cytofluorimetrie, přičemž vazba specifických monoklonálních protilátek byla vizualizována polyklonálními protilátkami proti myšímu případně potkanímu Ig značených fluorochromy (PE a FITC). V thymu již 51. den gestace byly prokázány jak $CD4^+$, tak $CD8^+$ T lymfocyty. Jejich množství v průběhu dalšího prenatálního vývoje průkazně narůstá a to jak v relativním, tak absolutním vyjádření. Vyneseme-li tento nárůst jako funkci času s následnou interpolací, je možno předpokládat expresi $CD4^+$, $CD8^+$ fenotypu u thymových lymfocytů kolem 40. dne gestace, tj. v období, kdy v tomto orgánu dosud není patrna jeho morfologická diferenciace na kůru a dřeň.

Ve slezině 51. den gestace jsou přítomny pouze lymfocyty $CD2^+$. Exprese fenotypu $CD4^+$ a $CD8^+$ byla pozorována až 60. den prenatálního vývoje. Jejich počet jak v absolutním, tak relativním vyjádření jako funkce času výrazně narůstá a představuje rozdíl tří řádů. Mízní uzliny byly sledovány pouze od 90. dne gestace, přičemž T lymfocyty v tomto sekundárním lymfatickém orgánu mají vyšší percentuální zastoupení než ve slezině. $CD4^+/CD8^+$ index ve slezině a thymu v průběhu vývoje postupně klesá až na hodnotu 1 s mírnou dominancí $CD4^+$ lymfocytární subpopulace. V mízních uzlinách prasečích fétů můžeme naopak sledovat dominanci $CD8^+$ subpopulace (index 0.85).

V postnatálním období jsme prokázali v periferní krvi průkazný nárůst cytotoxických $CD8^+$ lymfocytů, přičemž index $CD4^+/CD8^+$ klesá z hodnoty 1 až na 0,2 28. den gestace. Tato charakteristika lymfoidních subpopulací v cirkulující krvi je typická rovněž pro dospělý věk. Počet B lymfocytů s IgM receptorem v cirkulující krvi naopak od 90. dne prenatálního vývoje až do 28. dne postnatálního života postupně narůstá a to jak v relativním zastoupení (%), tak i při přepočtu na 1 litr krve.

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