Alterations in lymphocyte subpopulations in peripheral blood at manifestation of type 1 diabetes mellitus in childhood

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Zmeny lymfocytových subpopulácií v periférnej krvi pri manifestácii diabetes mellitus typu I v detskom veku

Abstract

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Background: **Alterations in cellular immunity at manifestation of type 1 diabetes mellitus, as described in publications so far, are equivocal. Moreover, the age of children was usually not taken into account.**
Objectives: **Exact inferentially statistical measures were used to arrive at reliable information.**
Methods: **Thirty four diabetic children and 48 normals were taken randomly according to the established criteria, and scrutinized. Lymphocyte subpopulations counts were measured by flow cytometry using three-color-labelled monoclonal antibodies against cell surface markers. The resulting absolute cell counts as well as percentages from the total lymphocyte count were expressed in terms of univariate and bivariate 95 % confidence intervals. They render an illustrative way for defining statistically significant (alpha = 5 %) differences between health and disease.**
Results: **The CD8, CD16 absolute counts in younger diabetics were significantly decreased in average to 96—58 % of the normal subgroup. For older children, CD4, CD8, CD16 and CD19 absolute counts were significantly lowered to 75—61 % of the norm. Relative changes in Ly subpopulations were less pronounced. The immunoregulatory index increased significantly to 125—128 % of the norm in either age group. The proportion of CD4 memory cells from the total of naive and memory cells was significantly increased to 122—133 % of the norm in diabetic children of either age group.**
C*onclusion*: More significant changes of lymphocyte subpopulations than those given in literature were revealed at manifestation of childhood type 1 diabetes. They testify to the

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Abstrakt

Michalková D., Mikulecký M., Tibenská E.:
Zmeny lymfocytových subpopulácií v periférnej krvi pri manifestácii diabetes mellitus typu I v detskom veku
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Pozadie problému: Údaje v literatúre o zmenách v bunkovej imunite pri manifestácii diabetes mellitus typ I sú nejednoznačné. Navyše neprihliadajú na vek detí.
Cieľ: Na získanie presných a spoľahlivých informácií sa použili inferenčné štatistické merania.
Metódy: Náhodný výber a prešetrenie 34 diabetických a 48 zdravých detí sa uskutočnil na základe stanovených kritérií. Subpopulácie lymfocytov sa počítali prietokovou cytometriou použijúc trojfarebne označené monoklonálne protilátky proti markerom bunkového povrchu. Výsledný absolútny počet lymfocytov sa vyjadril pomocou univarantných a bivarantných 95 % konfidenčných intervalov, ktoré dávajú možnosť ilustratívneho definovania štatisticky signifikantných rozdielov (alfa = 5 %) medzi zdravými a chorými deťmi.
Výsledky: Absolútne počty CD8 a CD16 u mladších diabetikov boli signifikantne zvýšené v priemere na 96—58 % zdravej podskupiny. U starších detí boli absolútne počty CD4, CD8, CD16 a CD19 signifikantne znížené na 75—61 % normy. Relatívne zmeny v subpopulácii lymfocytov boli menej výrazné. Imunoregulačný index sa signifikantne zvýšil na 125—128 % normy v oboch vekových skupinách. Celkový podiel pamäťových buniek CD4 na celkovom počte naivných a pamäťových buniek bol signifikantne zvýšený na 122—133 % normy v oboch vekových skupinách detských diabetikov.
Záver: V porovnaní s literatúrou sa zmeny v subpopulácii lymfocytov zistili pri manifestácii diabetu typu I v detskom veku významnejšie a potvrdzujú autoimunologickú patogenézu diabetes mellitus typ I. *(Tab. 3, obr. 3, lit. 18.)*

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autoimmune pathogenesis of the type 1 diabetes mellitus. ***(Tab. 3, Fig. 4, Ref. 18.)***
Key words: children, type 1 diabetes mellitus, manifestation, age, lymphocyte subpopulations.

Kľúčové slová: deti, diabetes mellitus typ I, manifestačný vek, subpopulácia lymfocytov.

In type 1 diabetes mellitus (DM 1), numerous changes in the cellular as well humoral immune response have been identified (Atkinson, 1992). The prediabetic stage was defined as an active autoimmune process with pancreatic B-cells destruction, preceding years of the onset of frank hyperglycemia (Faustman et al., 1991). This discovery gives hope for non-invasive screening of the early disease stage (Faustman et al., 1989), and even for preventing the onset of DM 1 by isohormonal therapy (Petersen et al., 1999). Different immunological changes were described for newly diagnosed DM 1 before the first insulin injection (Rodier et al., 1984; Petersen et al., 1999) and after it (Faustman et al., 1989). Another pecularities of the immune response were found in long-term DM 1 (Faustman et al., 1989; Smerdon et al., 1993). The immunological findings obtained by a snapshot of the prolonged process — at the disease manifestation — are often controversary, may be due to selection of patients, regimen and methods of analysis. The present paper addresses the issue of T and B lymphocytes subsets at the onset of DM 1 in younger and older children.

Patients and methods

Patients. 34 diabetic children: 11 aged 0—9 years (5 boys and 6 girls) and 23 aged 10—15 years (6 girls and 17 boys) were taken into the study. The normal group was recruited from 48 children (26 aged 0—9 years, 22 aged 10—15 years). The election of probands was random in each subgroup. The children with diabetes were investigated immediately after the diagnosis has been based on hyperglycemia and ketoacidosis before the first injection of insulin. Lymphocyte phenotyping. Lymphocyte subpopulations were examined from full peripheral blood (with EDTA), by flow cytometer (ORTHO). Three-colour fluorescence analysis was based using monoclonal antibodies against the surface markers (Buc et al., 1998). All used monoclonal antibodies used were directly conjugated mouse anti-human monoclonal antibodies: CD45RA (FITC), CD45RO(PE), CD4 (Per CP), CD3 (Per CP), CD4(FITC)/CD8(PE) (Becton-Dickinson Immunocytometry Try Systems) and CD16 (FITC)/CD19 (PE)/CD3(CyP) (Ortho Diagnostic Systems Inc.). The staining was performed using no wash protocol. This procedure offers the possibility to analyse the absolute counts of lymphocytes. In addition to absolute counts per microliter for total T-lymphocytes ($CD3^+$), T helpers ($CD3^+CD4^+$), T cytotoxic/suppressors ($CD3^+CD8^+$), NK cells ($CD16^+$), B lymphocytes ($CD19^+$) also their percentage proportions from total lymphocytes were calculated. Mutual proportion of $CD4^+CD45RA^+$ (naive cells) and $CD4^+CD45RO^+$ (memory cells) markers was determined as percentage where the total of 100 % represents was the sum of both naive and memory cells. The double positive cells, corresponding with low levels of expression of $CD45RA^+RO^+$, were arbitrarily — according to the level of expression of either antigen — added to RA^+ or RO^+ phenotype. Statistical analysis is based on the R.A. Fisher's concept of interval estimates rather than p-values (Brown, 1983; Evans et al., 1988). Both univariate and bivariate 95 % confidence intervals for separate subgroups/subpopulations and for some differences betwe-

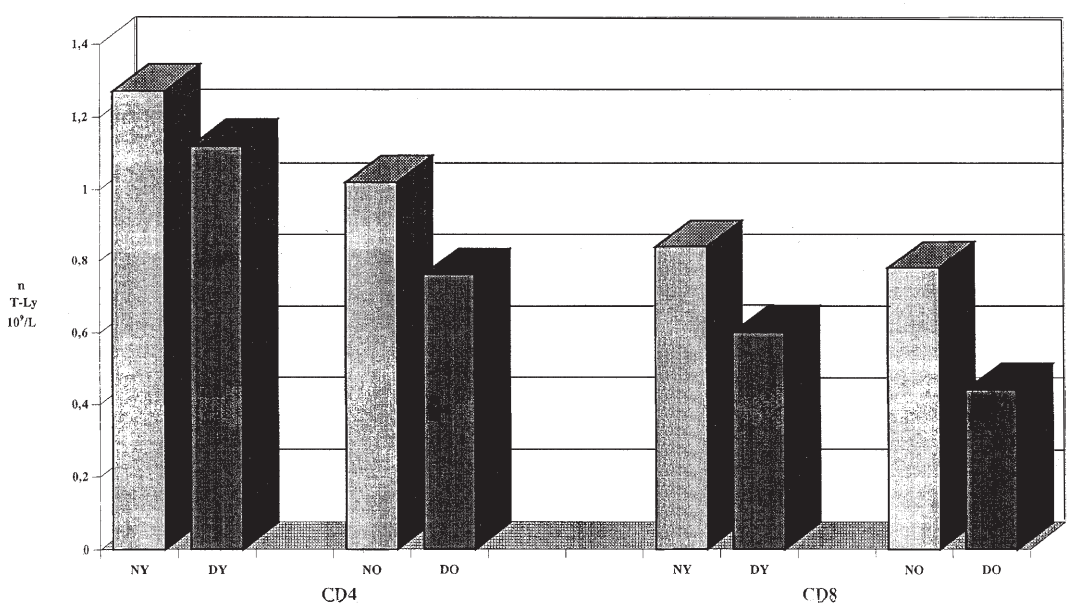

Fig. 1. Univariate representation of mean CD4 and CD8 absolute counts in separate subgroups (NY = normal younger children, DY = diabetic younger children, NO = normal older children, DO = diabetic older children).

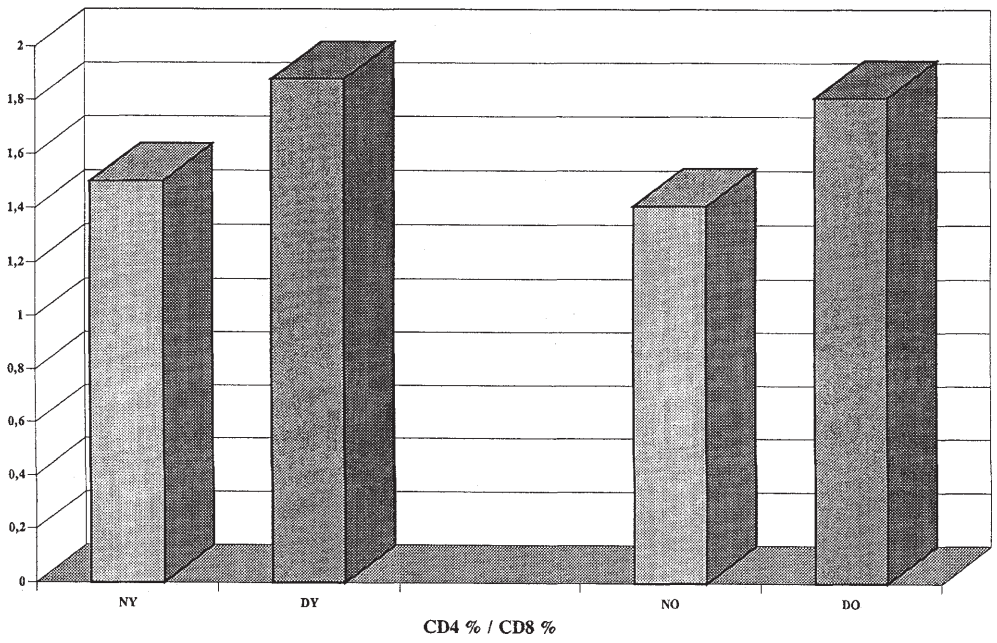

Fig. 2. Immunoreactive index (IRI) in normal younger children, diabetic younger children, normal older children and diabetic older children.

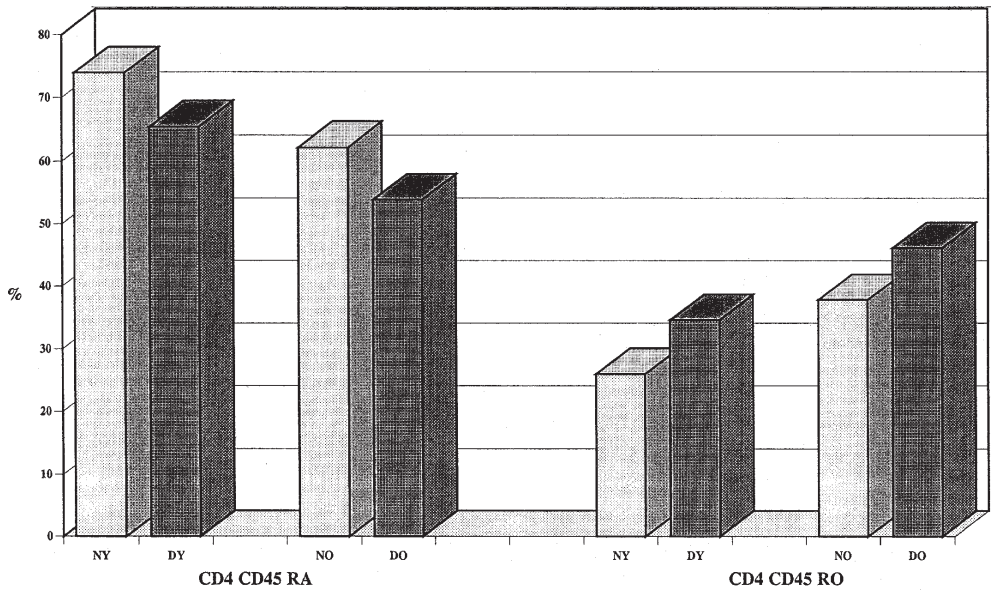

Fig. 3. Univariate representation of mean CD4 naive and memory cells in separate subgroups (NY = normal younger children, DY = diabetic younger children, NO = normal older children, DO = diabetic older children).

en them were computed. For univariate statistics, the parametric or nonparametric (Campbell et al., 1988) procedure of calculation was used, according to the result of normality testing. The non-overlapping between given intervals substantiates the 5 % significance level for corresponding difference. This procedure makes it also possible to compare our results reasonably with those given in literature.

Results

The basic results are shown in Table 1 and Figures 1—4. Table 1 displays the absolute counts of total lymphocytes, their ratio and, for naive and memory cells, their proportions. Besides, also the differences are shown in units of measurement and as percentage change. The

Tab. 1. Means with 95 % confidence intervals (in parentheses) for indicators of cellular immunity in young (Y) and old (O) normal (N) and diabetic (D) children. Also differences D-N are evaluated ([n]nonparametrically).
Tab. 1. Priemery ([m] — mediány, pre výbery so zamietnutím hypotézy o normalite) s 95 % konfidenčnými intervalmi (v zátvorkách) pre niektoré indikátory bunkovej imunity u mladších (Y) a starších (O) normálnych detí (N) a pacientov s diabetom (D). Vyhodnotené sú aj rozdiely D-N ([n] — neparametricky).

Parameter	Group	N	D	D-N	Relative change %
Ly	Y	3.12[m]	2.74	-0.58[n*]	-19*
		(2.83; 3.82)	(2.13; 3.36)	(-1.25; -0.16)	(-40 -5)
	O	2.62[m]	1.92	-0.75[n*]	-29*
		(2.46; 2.90)	(1.64; 2.20)	(-1.15; -0.38)	(-44 -15)
CD4	Y	1.27	1.12	-0.15	-12
		(1.17; 1.37)	(0.83; 1.41)	(-0.43; +0.13)	(-34 +10)
	O	1.02	0.76	-0.26*	-25*
		(0.92; 1.12)	(0.62; 0.90)	(-0.43; -0.09)	(-42 -9)
CD8	Y	0.84	0.60	-0.24*	-29*
		(0.77; 0.92)	(0.46; 0.75)	(-0.39; -0.09)	(-46 -11)
	O	0.72	0.44	-0.28*	-39*
		(0.65; 0.80)	(0.37; 0.52)	(-0.38; -0.18)	(-53 -25)
CD16	Y	0.38	0.22	-0.16*	-42*
		(0.33; 0.43)	(0.14; 0.30)	(-0.25; -0.07)	(-66 -18)
	O	0.28	0.18	-0.11*	-39*
		(0.25; 0.32)	(0.14; 0.21)	(-0.16; -0.06)	(-57 -21)
CD19	Y	0.48	0.45[m]	-0.02[n]	-4
		(0.43; 0.54)	(0.39; 0.63)	(-0.10; +0.10)	(-21 +21)
	O	0.39	0.27[m]	-0.10[n]*	-26*
		(0.35; 0.44)	(0.24; 0.36)	(-0.16; -0.02)	(-41 -5)
CD3m	Y	26.7	32.8[m]	+6.4[n]	
		(23.1; 30.4)	(27.0; 35.7)	(-2.0; +12.7)	
	O	40.9	43.9	+3.0	
		(35.9; 46.0)	(39.5; 48.3)	(-3.4; +9.3)	
CD4m	Y	26.0	34.6	+8.6*	
		(22.7; 29.2)	(29.1; 40.1)	(+2.8; +13.5)	
	O	37.8	46.1	+8.3*	
		(33.8; 41.8)	(40.6; 51.6)	(+1.8; +14.8)	
IRI	Y	1.50	1.88	+0.38*	+25*
		(1.43; 1.57)	(1.47; 2.28)	(+0.02; +0.74)	(+1 +49)
	O	1.41	1.81	+0.40*	+28*
		(1.34; 1.49)	(1.59; 2.04)	(+0.17; +0.63)	(+12 +45)
		Percentage fr	om the total of	RA and RO	
$CD_4CD_{45}RO$	Y	26.0	34.6	+8.6*	+33*
		(22.7; 29.2)	(29.1; 40.1)	(+2.8; +13.5)	(+11 +45)
	O	37.8	46.1	+8.3*	+22*
		(33.8; 41.8)	(40.6; 51.6)	(+1.8; +14.8)	(+5 +39)

* $p<0.05$, [m] medians, for samples with rejected normality hypothesis

former ones are the most informative. All absolute cell counts were at the onset of DM 1 decreased, except for CD4 and B-cells in younger subgroup significantly. This decrease was, except NK cells, non-significantly more pronounced in older children. The same holds for significant increase of the immunoregulatory index in either gender, explained by a more pronounced decrease in CD8 cells compared with that in CD4 cells (Figs 1, 2). The significant increase in the proportion of memory cells is non-significantly more pronounced in younger diabetic children (Fig. 3). The differences between the norm and diabetes at its manifestation in either age group are obvious also from Figure 4 for selected parameters. The 95 % confidence ellipses were constructed for absolute, as well as for relative (%) values. Non-overlapping parts of them are shadowed and represent areas of both parameters' values combinations which are typical for the given subgroup. On the other hand, the overlapping parts of ellipses are white; they belong to two or more subgroups. Maximal number of possible overlappings between 4 subgroups is six. Table 2 gives these numbers both for absolute and relative values from Figure 4. Accordingly, absolute counts of CD4 versus CD8 and CD16 versus CD19 allow a perfect recognition of diabetes against the norm in both age groups. This, however, is not true for percentage values. The younger subgroup differs clearly from the older one in healthy children, not in diabeties. The most important numerical results for percentages are included in Table 3 for comparison with those in absolute numbers, as well as with percentage data from literature. An equivocal significant depression is seen for CD8 both in absolute and relative terms. The fall in

Tab. 2. Overlapping (+) or nonoverlapping (-) between subgroups (NY — normal younger, DY — diabetic younger, NO — normal older, DO — diabetic older) for some lymphocyte subpopulations (absolue counts and percentages). Minimal overlapping in brackets.
Tab. 2. Prekrývanie (+) alebo neprekrývanie (-) medzi podskupinami (NY — normálne mladšie, DY — diabetické mladšie, NO — normálne staršie, DO — diabetické staršie) pre niektoré lymfocytové subpopulácie (absolútne počty a percentá). Minimálne prekrývanie v zátvorkách).

	CD4 versus CD8		CD16 versus CD19	
	abs.	%	abs.	%
NY-DY	–	+	–	+
NY-NO	–	+	–	+
NY-DO	–	–	–	(+)
DY-NO	(+)	+	–	+
DY-DO	+	+	+	+
NO-DO	–	–	–	(+)
Total	2	4	1	6

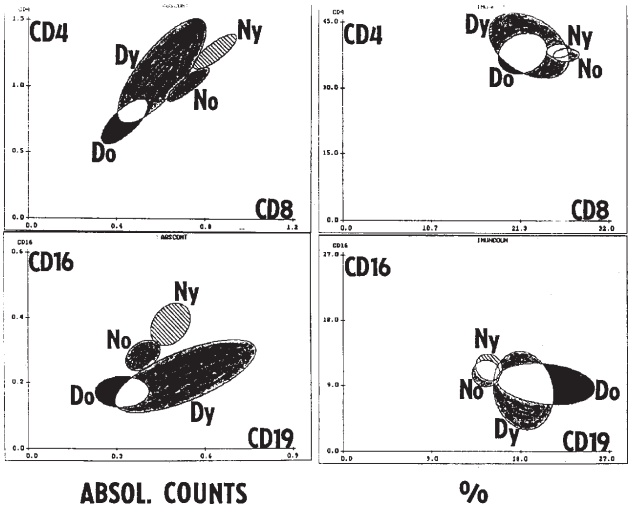

Fig. 4. Bivariate plots for correlation between CD4 and CD8 (top) and CD16 and CD19 (bottom) lymphocytes in subgroups as in Fig. 1. Left panel: absolute counts in thousands per microliter of blood. Right panel: percentages from the total of all lymphocytes. The 95 % confidence ellipses delineate areas where combinations of mean estimates for either variable and given subgroup can with 95 % probability oscillate.

NK cells is significant only for their absolute counts. No changes in DM 1 were seen for CD4 when expressed in percentage. Finally, the decrease in absolute numbers of B cells in diabetes, for older children significant, turned to an increase in their percentage, significant for both age groups, obviously due to a more pronounced decrease in total lymphocyte counts in diabetic children.

Discussion

A survey of literature has revealed that outcomes of other studies are expressed usually in relative values — as percentage of the given lymphocyte subpopulations. That is why also our results are in Table 3 are shown in the same way, in addition to absolute counts of separate immune cells, for comparisons. The best agreement of our results with two other contributions has been achieved for B cells: significant increase in their percentage in DM 1 matches with two other contributions, in one case significantly. Also the diabetic changes in NK cells were unidirectional: always decreasing, however only our absolute counts are significant. The changes in CD4 and CD8 were studied more frequently by other authors with variable results. Table 3 allows also to hypothesize about the reasons of the latter variability. The heterogenous design of observations makes the homogeneity of the described samples. Thus, the age structure ranged from over adolescent children to adults up to 40 years of age. The male/female ratio was considerably different, too. Particularly important could be the broadly differing time which elapsed between the moment of diagnosis and examination: between the day of diagnosis and one year! Finally, in the majority, but not in all designs, the examination was done under insulin treatment. Its effect on circulating lymphocytes was interpreted as normalizing the total T cell defect and the T4/T8 ratio (Rodier et al., 1984) although the data presented by these authors do not support this view unequivocally. The present paper, as we hope, represents a well defined setting: children of two age groups examined immediately after the diagnosis and before starting the insulin therapy. Accordingly, our contribution can be compared more appropriately than those of other authors to a snapshot photograph. That has possibly decreased the variances and gave a better chance for significant results. Another interesting however problematic issue is that of naive and memory cells. It is not easy to compare our results, based on differentiation on $CD4^+CD45RA^+$ (naive, virgin, unprimed) and $CD4^+CD45RO^+$ (memory, primed) cells, with the contemporary knowledge recognizing changes in $CD4^+CD45RA^+RO^-$, -RA^+RO^+ and -RA^-RO^+ cells. It appears to be well established that in newly diagnosed DM 1 patients the RA^+RO^- and RA^-RO^+ cells proportions are decreased, while those of RA^+RO^+ (double positive) cells, corresponding with recently activated lymphocytes, is vastly increased (Peakman, 1997; Petersen et al., 1999; Al-Kassab et al., 1990). The increased memory cells proportion in our DM 1 patients obviously includes also the part of RA^+RO^+cells. CD45RO+ expression on CD4+ CD45RA+RO+ lymphocytes was higher in patients with the recently onset DM1 compared with control subjects (Smerdon et al., 1993). This is in good agreement with our results for the younger children (Tab. 1, Fig. 3). The novelty of the presented paper is threefold. Firstly, a significant influence of children's age on the results was demonstrated. Future studies should therefore respect the age of subjects. Secondly, the absolute counts appear to bear a more important information than the relative ones, in contradiction with general view and some results (Faustman et al., 1989). Thirdly, the multivariate processing of data, as usually, improved the chance to find significant differences: univariate interval estimates can overlap while multivariate ones (e.g. confidence ellipses) may not, as shown on Figure 4. The most pronounced outcome of the present study is the reduction in the cytotoxic/suppressoric phenotype (CD8) lymphocytes. This agrees with the classical theory of pathogenesis of autoimmune diseases as the depressed immunological suppressive functions trigger the autoagressive processes (Bach, 1988; Buc et al., 1994; Roitt, 1998).*

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Tab. 3. Changes in selected lymphocyte subpopulations counts in healthy children (N) and at DM 1 onset found by other authors and compared with those obtained in the present study. The percentages (%) refer to total lymphocyte counts.
Tab. 3. Zmeny v počte vybraných lymfocytových subpopulácií u zdravých detí (N) a pri manifestácii DM 1 zistené inými autormi v porovnaní s výsledkami našej štúdie. Percentá (%) sú odvodené od celkových lymfocytov.

Source	ILONEN et al.(1984)	RODIER et al.(1984)	SCHEININ et al.(1988)	FAUSTMAN et al.(1989)	BUSCHARD et al.(1990)	SMERDON et al.(1993)	HEHMKE et al.(1995)	The present study	
Patients								**Younger**	**Older**
Sample size	63	14	38	20	13	34	25	11	23
Age (years) (±SD)	8 ± 4	6 – 10	2 – 15	10 – 32	28 ± 11	21 ± 11	7 – 29	0 – 9	10 –15
Male / female	? / ?	7 / 7	22 / 16	? / ?	10 / 3	21 / 13	13 / 12	5 / 6	17 / 6
Onset definition (days since the day of dg)	≤ 7	≤ 30	7 – 14	2 – 5	1 – 7	≤ 365	≤ 7	1	1
Examination after starting insulin treatment	Yes	No	Yes	Yes	Yes	Yes	Yes	No	No
Lymphocyte subsets (N/DM 1)									
CD4	48%/44%	49%/47%	44%/51%	49%/53%	42%/43%	65%/63%	42%/43%	[a]1,3/1,1 (39%/40%)	[a]1,0/0,8* (38%/38%)
CD8	21%/26%	30%/26%	29%/28%	25%/24%	29%/24%	32%/34%	29%/28%	[a]0,8/0,6* (26%/22%)	[a]0,7/0,4* (27%/22%)
CD4 / CD8	2,3/1,6*	1,7/2,1	1,6/1,9*	2,2/2,2	1,5/1,8	2,3/2,1	1,5/1,6	1,5/1,9*	1,4/1,8*
CD16 (NK)	? / ?	? / ?	? / ?	? / ?	? / ?	? / ?	12%/10%	[a]0,4/0,2* (12%/8%)	[a]0,3/0,2* (11%/8%)
CD19 (B)	?/?	12%/18%	? / ?	? / ?	? / ?	? / ?	12%/13%	[a]0,5/0,4 (15%/18%)	[a]0,4/0,3* (15%/21%)

* $p \leq 0,05$ [a] counts in thousands per microliter

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