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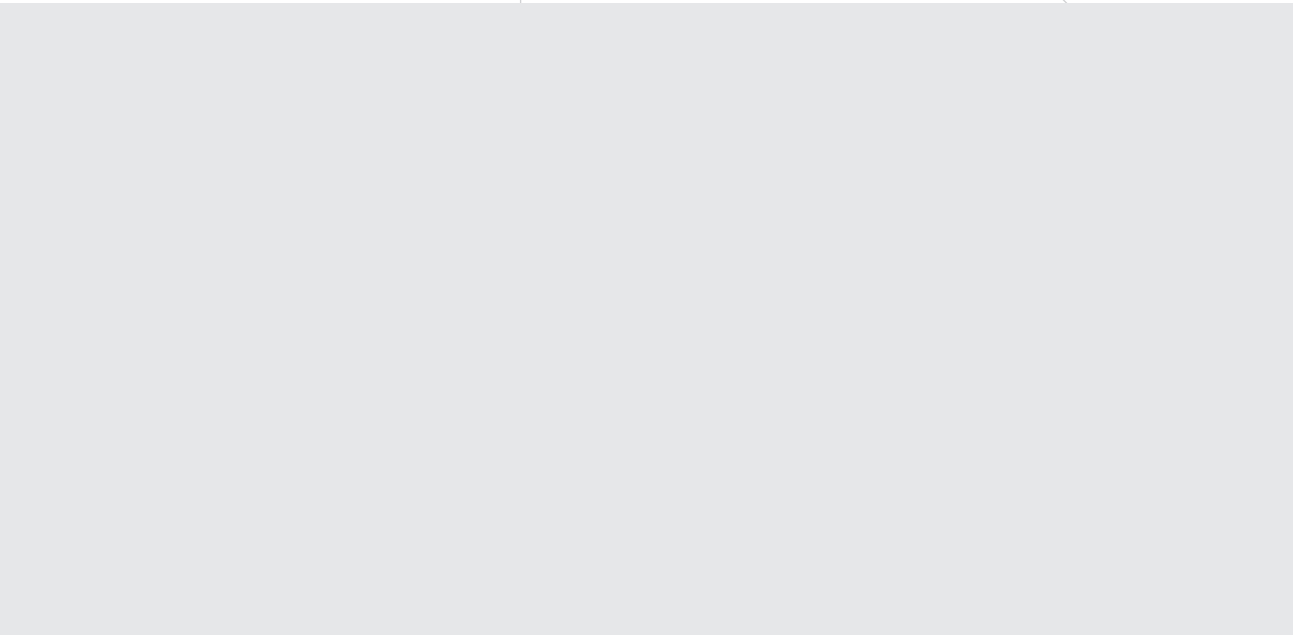


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Author: Kusters, Maria Antonia Adriana (Maaïke)

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**BOTH NORMAL MEMORY COUNTS
AND DECREASED NAIVE CELLS FAVOR
INTRINSIC DEFECT OVER EARLY
SENESCENCE OF DOWN SYNDROME
T-LYMPHOCYTES**

MAA Kusters, EFA Gemen, RHJ Verstegen, PC Wever, E de Vries

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ABSTRACT

Because of their increased malignancies, autoimmune diseases, and infections, patients with Down syndrome (DS) show features of immunodeficiency. The DS thymus and T-lymphocyte subsets have indeed proven to be different, and this has been interpreted as precocious ageing. Our study on T-lymphocyte subpopulations in DS shows that the normal expansion of naive helper- ($CD4^+CD45RA^+$) and cytotoxic ($CD8^+CD45RA^+CD27^+$) T-lymphocytes is lacking in the first years of life; which is more logically explained by an intrinsic T-lymphocyte defect. Furthermore, memory cell numbers are not different from age-matched controls (AMC), which do not support the hypothesis of precocious ageing. Although the absolute numbers of T-lymphocyte subpopulations approach AMC levels towards adulthood, the persistent clinical problems suggest that these cells may not function optimally. However, the clinical picture does not fit severe T-lymphocyte deficiency. The latter concept is also supported by our finding that cytomegalovirus (CMV)-seropositive DS children show similar numbers of terminally differentiated cytotoxic T-lymphocytes when compared to healthy children, instead of increased numbers as are seen in immunocompromised hosts.

INTRODUCTION

Down syndrome (DS) is associated with a high frequency of haematological malignancies [1-4], autoimmune diseases like celiac disease and hypothyroidism [5-7], as well as recurrent, mainly respiratory, infections [2, 8]. This fits with immunodeficiency. Indeed, the thymus in DS children is smaller and abnormal [9-13], and blood T-lymphocyte subpopulations differ from healthy controls [14-16]. This has been interpreted as precocious ageing of the immune system due to the lower relative number of CD4⁺CD45RA⁺ naive T-lymphocytes [17, 18] and lower T-cell-receptor excision circle counts [19, 20] in DS children. However, we recently showed [21] that the vast expansion of T-lymphocytes in the first years of life is abrogated, favoring an intrinsic defect. We studied T-lymphocyte subpopulations in DS children compared with age-matched controls (AMC) to analyze whether the results support this alternative theory.

T-lymphocyte differentiation and expansion are influenced by encountered viral infections. Especially, the expansion of CD45RA⁺CD27⁻ terminally differentiated cytotoxic T-lymphocytes (Tc), which is described as unique for cytomegalovirus (CMV) infection. The individual set-point is defined by the degree of immunocompetence during the primary CMV-contact: immunodeficient children show higher median absolute numbers of terminally differentiated Tc [22]. To further assess the degree of immuno(in)competence in DS, we related T-lymphocyte subpopulations to CMV-serostatus and compared the DS children with groups from the literature with different immune status during primary CMV-contact [22, 23].

METHODS

Study population

An extra 3 ml of EDTA blood and 7 ml of blood without additive was drawn from 95 non-institutionalized DS children (49 males; mean age 7 years, range 1-20) visiting the Jeroen Bosch Hospital, 's-Hertogenbosch, or the Rijnstate Hospital, Arnhem, The Netherlands, during routine follow-up of thyroid function after parental informed consent. All children were otherwise healthy at the time of sampling. Leftover EDTA blood from 33 healthy AMC children who underwent venipuncture for e.g. pre-operative screening for minor surgery, was used as control. The study was approved by the local Medical Ethics Committees of all participating hospitals.

We divided the children into the same age groups that were used in a large Dutch reference study analyzing lymphocyte subpopulations [24]. Absolute and relative numbers of T-lymphocyte subpopulations were compared in DS and AMC children. Absolute numbers of terminally differentiated Tc of CMV-seropositive (CMV⁺) DS

children were compared with CMV-seronegative (CMV) DS children and with the results from the evaluation and discussion from recent literature [22, 23] including children with human immunodeficiency (HIV) infection, children using immunosuppressive therapy and children who were otherwise healthy at the time of primary CMV-contact.

Immunophenotyping

Three-color flow cytometric immunophenotyping was performed to determine T-lymphocyte subpopulations in both DS and AMC using the lysed whole-blood method. FITC-, phycoerythrin (PE)- and PE-cyanin 5 (PE-Cy5)-conjugated MAb were used with the following antigen specificity: CD3 (PE-Cy5; Immunotech, Marseille, France), CD3/CD4 (FITC/PE; IQProducts, Groningen, The Netherlands), CD8 (PE-Cy5; Immunotech), CD14 (PE; Beckton Dickinson (BD), San Jose, CA, USA), CD15 (FITC; IQ Products), CD16/CD56 (FITC; BD), CD19 (PE-Cy5; Immunotech), CD27 (FITC; BD), CD45 (PE-Cy5; Immunotech), CD45RA (PE; Coulter Immunology, Hialeah, USA), CD45RO (FITC; Serotec DPC, Apeldoorn, The Netherlands), TCR- $\alpha\beta$ (PE-Cy5;BD) and TCR- $\gamma\delta$ (PE; BD).

Aliquots were incubated for 15 minutes at room temperature with different combinations of optimally titrated conjugated MAb to determine the following lymphocyte subpopulations: T-lymphocytes (CD3⁺), B-lymphocytes (CD19⁺), natural killer (NK) cells (CD16⁺and/orCD56⁺CD3⁻), helper-T-lymphocytes (Th) (CD3⁺CD4⁺), Tc (CD3⁺CD8⁺), naive Th (CD3⁺CD4⁺CD45RA⁺), memory Th (CD3⁺CD4⁺CD45RO⁺), naive Tc (CD8⁺CD45RA⁺CD27⁺), central memory Tc (CD8⁺CD45RA⁻CD27⁺), effector memory Tc (CD8⁺CD45RA⁺CD27⁻), terminally differentiated Tc (CD8⁺CD45RA⁺CD27⁻), CD3⁺TCR- $\alpha\beta$ ⁺ and CD3⁺TCR- $\gamma\delta$ ⁺ T-lymphocytes. Erythrocytes were lysed using FACSLysing solution (BD) according to the manufacturer's protocol. The remaining cells were washed twice with PBS with BSA and analyzed by flow cytometry after calibration with the SPHERO CaliFlow kit (Spherotech, Libertyville, IL, USA) as recommended by the European Working Group on Clinical Cell Analysis [25]. A FACScan or FACSCalibur flow cytometer (BD) was used. Absolute leukocyte counts were determined with a Sysmex SE-9500 hematology analyzer (Sysmex, Kobe, Japan). The lymphocyte gate was checked with a CD15/CD14/CD45 triple labeling and considered correct if <5% contamination was present. B-lymphocytes and NK cells were used to check whether the T+B+NK equaled $100 \pm 5\%$. Absolute numbers of lymphocyte subpopulations were calculated by multiplying the absolute leukocyte count ($\times 10^9/l$) by the relative total lymphocyte size (%) and relative size of the lymphocyte subpopulation (%).

CMV serology and PCR

Anti-CMV IgM and IgG were measured in duplo by enzyme-linked fluorescence analysis using the VIDAS test in 90 DS children (Biomerieux, Lyon, France); for 5 DS

children, serum was not available. IgG avidity tests (VIDAS, Biomerieux) were performed to differentiate between recent (<3 months) and late CMV contact. Real-time PCR for CMV-DNA [22] was performed in CMV-IgG⁺ children to differentiate between active and latent infection.

Review of medical files

The medical files of 91/95 DS children were reviewed retrospectively; four files were unavailable. The 91 children were divided into four groups: 1) no increased infection rate, 2) increased infection rate (age at inclusion <8 years), 3) increased infection rate (age at inclusion >8 years), and 4) increased infection rate until, but not after the age of 8 years. In addition, the presence of celiac disease or autoimmune hypothyroidism was noted.

Statistical analysis

To compare the T-lymphocyte subpopulations between DS and AMC the Mann Whitney U-test was used ($p < 0.05$). An analysis of variance (completely randomized two-factorial design; $p < 0.05$) was applied to the data to test the overall effects of age (2-16 years) and group (DS and AMC). Age groups with low numbers of AMC children were excluded (<2 yr; >16 yr) from this analysis. Levene's test for equality of error variances was used on all subpopulations ($p < 0.05$). Tc subpopulations of CMV-seronegative (CMV⁻) and CMV⁺ DS children were analyzed after log transformation by *t* test ($p < 0.05$). All analyses were performed with SPSS 16.0 for Windows.

RESULTS

The absolute and relative numbers of the analyzed CD3⁺ T-lymphocyte subpopulations, and the results of the statistical analyses are listed in table 1 (the values for CD3⁺ T-lymphocytes, CD3⁺CD4⁺ Th, and CD3⁺CD8⁺ Tc were reported before [21]). None of the interaction effects were significant. This means that the effects of age on the various T-lymphocyte subpopulations do not differ between DS children and AMC children; although T-lymphocytes and Th lack the expansion normally seen in the first years of life [24], the overall pattern seen in time is the same in DS and AMC. Clinically relevant data is presented in Table 2. We did not find a relation between any of the determined T-lymphocyte subpopulations and the incidence of infections or autoimmune diseases in these DS children.

Naive and memory CD3⁺CD4⁺ Th

The absolute numbers of CD45RA⁺ naive Th are reduced in DS children. Naive Th lack the expansion seen in AMC children during the first years of life, but the overall pattern

Table 1 Absolute and relative numbers of T-lymphocyte subpopulations.

		9–15 months		15–24 months		2–5 years		
T-lymphocytes	DS	1.94 (0.98-4.29)	n=11	2.10 (1.22-2.79)	n=8	1.54 (0.81-3.07)	n=16	
		69 (58-81)	n=11	73 (66-81)	n=8	74 (65-84)	n=16	
	AMC	5.00	n=1	2.30	n=1	1.95 (1.20-2.90)	n=10	
		70	n=1	60	n=1	66 (59-71)	n=10	
Th	DS	1.22 (0.69-2.97)		1.36 (0.70-1.59)		0.78 (0.04-1.67)		
		69 (45-79)		62 (47-78)		55 (41-71)		
	AMC	3.30		1.40		1.15 (0.70-2.00)		
		65		60		60 (52-69)		
Tc	DS	0.67 (0.20-1.20)		0.76 (0.38-1.32)		0.54 (0.28-1.41)		
		30 (20-41)		36 (19-50)		41 (26-58)		
	AMC	1.40		0.80		0.70 (0.50-1.10)		
		29		33		35 (29-47)		
Th naive	DS	0.91 (0.50-1.90)		0.90 (0.39-1.12)		0.49 (0.19-1.15)		
		73 (63-83)		68 (55-72)		58 (37-70)		
	AMC	2.80		1.16		0.82 (0.41-1.60)		
		86		83		70 (58-80)		
Th memory	DS	0.22 (0.13-0.72)		0.25 (0.16-0.35)		0.27 (0.14-0.39)		
		18 (10-28)		23 (16-33)		32 (22-50)		
	AMC	0.36		0.17		0.29 (0.18-0.38)		
		11		12		23 (16-38)		

	5-10 years		10-16 years		>16 years		DS vs AMC (2-16 years) *	Age effect (DS+AMC; 2-16 years) **
	1.39 (0.62-2.67)	n=38	1.00 (0.54-2.45)	n=19	1.25 (1.06-1.56)	n=3	p=0.003	p<0.001
	74 (58-89)	n=38	69 (59-85)	n=19	74 (72-81)	n=3	p<0.001	NS
	1.85 (1.50-2.40)	n=8	1.60 (0.80-2.40)	n=11	1.40 (1.10-1.70)	n=2		
	69 (58-72)	n=8	65 (59-77)	n=10	69 (63-75)	n=2		
	0.63 (0.30-1.40)		0.59 (0.28-1.16)		0.81 (0.48-0.92)		p<0.001	p<0.001
	46 (25-65)		58 (31-65)		52 (45-73)		p<0.001	NS
	1.00 (0.90-1.60)		0.80 (0.50-1.20)		0.95 (0.80-1.10)			
	65 (49-71)		56 (41-73)		70 (68-71)			
	0.70 (0.27-1.45)		0.45 (0.18-1.15)		0.51 (0.33-0.78)		p<0.001	NS
	47 (33-71)		40 (29-59)		48 (27-51)		p<0.001	NS
	0.50 (0.40-0.80)		0.50 (0.30-1.00)		0.40 (0.30-0.50)			
	29 (26-45)		36 (22-49)		29			
	0.28 (0.07-0.97)		0.20 (0.01-0.43)		0.19 (0.06-0.29)		p<0.001	p<0.001
	44 (11-69)		37 (2-59)		24 (7-60)		p<0.001	p<0.001
	0.73 (0.64-1.30)		0.54 (0.27-0.80)		0.57 (0.44-0.70)			
	72 (64-80)		61 (54-67)		59 (55-64)			
	0.27 (0.12-0.51)		0.25 (0.15-0.65)		0.50 (0.15-0.66)		NS	NS
	45 (21-74)		52 (30-70)		62 (31-72)		p<0.001	p<0.001
	0.24 (0.14-0.39)		0.25 (0.12-0.44)		0.31 (0.27-0.43)			
	23 (15-30)		31 (28-39)		32 (31-34)			

Table 1 Continued.

		9–15 months	15–24 months	2–5 years	
Tc naive	DS	0.45 (0.11-0.83)	0.26 (0.14-0.63)	0.29 (0.09-0.85)	
		72 (57-82)	53 (19-82)	47 (18-86)	
	AMC	0.64	0.69	0.53 (0.27-0.71)	
		46	86	66 (49-89)	
Tc centr mem	DS	0.15 (0.04-0.34)	0.13 (0.06-0.26)	0.19 (0.06-0.91)	
		23 (15-41)	23 (8-35)	39 (11-65)	
	AMC	0.53	0.10	0.16 (0.08-0.32)	
		38	12	28 (11-33)	
Tc eff mem	DS	0.01 (0.00-0.02)	0.04 (0.00-0.54)	0.06 (0.01-0.61)	
		1 (0-3)	9 (0-45)	4 (1-34)	
	AMC	0.17	0.01	0.01 (0.00-0.14)	
		12	1	2 (0-13)	
Tc term diff	DS	0.01 (0.00-0.06)	0.02 (0.00-0.21)	0.01 (0.00-0.32)	
		2 (0-9)	3 (0-18)	1 (0-31)	
	AMC	0.05	0.01	0.00 (0.00-0.11)	
		3	1	2 (0-10)	
CD3⁺TCRαβ⁺	DS	1.89 (0.91-3.75)	1.94 (1.15-2.65)	1.44 (0.66-2.69)	
		93 (77-98)	95 (90-96)	91 (68-97)	
	AMC	4.83	2.13	1.79 (1.07-2.76)	
		97	93	92 (86-97)	

	5–10 years	10–16 years	>16 years	DS vs AMC (2-16 years) *	Age effect (DS+AMC; 2-16 years) **
	0.23 (0.03-0.62)	0.16 (0.05-0.55)	0.11 (0.08-0.15)	p=0.001	p<0.001
	42 (10-74)	41 (13-69)	31 (11-31)	p<0.001	NS
	0.34 (0.23-0.54)	0.25 (0.16-0.64)	0.23 (0.15-0.30)		
	68 (39-77)	57 (37-80)	54 (49-59)		
	0.23 (0.09-0.65)	0.14 (0.07-0.50)	0.23 (0.20-0.25)	p=0.012	NS
	37 (12-62)	37 (20-60)	50 (30-62)	p=0.001	NS
	0.13 (0.07-0.31)	0.12 (0.07-0.23)	0.13 (0.09-0.17)		
	26 (17-56)	32 (13-39)	32 (30-34)		
	0.09 (0.01-0.61)	0.07 (0.01-0.68)	0.09 (0.03-0.70)	p=0.001	NS
	8 (1-42)	7 (2-48)	10 (3-52)	p=0.008	NS
	0.02 (0.01-0.38)	0.02 (0.01-0.16)	0.03 (0.01-0.04)		
	4 (2-6)	7 (1-22)	9 (2-15)		
	0.03 (0.01-0.38)	0.02 (0.00-0.21)	0.04 (0.01-0.06)	NS	NS
	5 (1-41)	8 (1-27)	7 (3-8)	p=0.044	NS
	0.02 (0.00-0.06)	0.02 (0.00-0.09)	0.02 (0.02-0.03)		
	3 (1-10)	4 (2-10)	5 (5-5)		
	1.20 (0.57-2.49)	0.91 (0.49-2.12)	1.19 (0.87-1.36)	p=0.001	p=0.001
	89 (61-95)	91 (74-98)	88 (82-96)	NS	NS
	1.64 (1.38-2.28)	1.42 (0.74-2.17)	1.34 (1.05-1.63)		
	93 (84-98)	89 (80-96)	96 (96-96)		

Table 1 Continued.

		9–15 months	15–24 months	2–5 years	
CD3 ⁺ TCRγδ ⁺	DS	0.17 (0.03-0.52)	0.11 (0.05-0.18)	0.13 (0.04-0.53)	
		7 (2-23)	5 (3-10)	9 (3-32)	
	AMC	0.16	0.17	0.14 (0.07-0.33)	
		3	7	8 (3-14)	

Grey shaded areas: absolute numbers of T-lymphocyte subpopulations (10⁹ cells/l), blank background: relative numbers (%); AMC = age-matched control children; DS = Down syndrome children; NS = not significant; centr mem = central memory; eff mem = effector memory; term diff = terminally differentiated * Analysis of variance, effect of group (children aged 2-16 years).** Analysis of variance, DS and AMC children together (2 to 16 years), effect of age.

seen in time is the same in both DS children and AMC children. Despite increased relative numbers of CD45RO⁺ memory Th, absolute numbers do not differ from AMC children due to the lower absolute counts of total Th in DS children. The memory Th subset does not show an age-related change in size in either DS or AMC children (Figure 1).

Naive, central memory, effector memory and terminally differentiated CD8⁺ Tc

Like naive Th, the absolute numbers of CD45RA⁺CD27⁺ naive Tc are decreased in DS, and lack the normal expansion seen in AMC during the first years of life, although the overall pattern seen in time is the same in DS and AMC. The absolute numbers of both CD45RA⁺CD27⁺ central memory and CD45RA⁺CD27⁻ effector memory Tc are higher in DS than in AMC, but values differ widely (Levene's test: p=0.009 and p=0.003, respectively). The absolute numbers of CD45RA⁺CD27⁻ terminally differentiated Tc are low in all age groups in both DS as well as AMC children. Neither in memory Th, the memory Tc subsets show an age-related change in size in DS or AMC (Figure 2).

TCR-αβ⁺ and TCR-γδ⁺ CD3⁺T-lymphocytes

The absolute number of TCR-γδ⁺ T-lymphocytes in DS children is lower, but values widely differ in AMC children (Levene's test: p=0.000). As was expected, the absolute numbers of TCR-αβ⁺ T-lymphocytes follow the pattern of total T-lymphocytes.

	5–10 years	10–16 years	>16 years	DS vs AMC (2-16 years) *	Age effect (DS+AMC; 2-16 years) **
	0.14 (0.04-0.66)	0.09 (0.02-0.33)	0.18 (0.04-0.18)	p=0.043	NS
	11 (5-39)	9 (2-26)	12 (3-17)	NS	NS
	0.12 (0.03-3.24)	0.17 (0.06-1.48)	0.06 (0.05-0.06)		
	7 (2-16)	11 (4-19)	4 (4-5)		

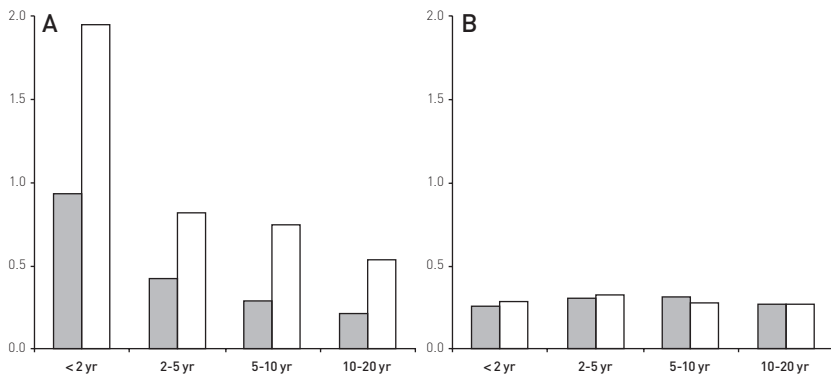


Figure 1 Median absolute numbers ($\times 10^9/l$) of helper-T-lymphocytes per age group.

- A. Naive helper-T-lymphocytes (CD₃⁺CD₄⁺CD₄₅RA⁺)
- B. Memory helper-T-lymphocytes (CD₃⁺CD₄⁺CD₄₅RO⁺).

Grey bars: Down syndrome children; white bars: age-matched reference values.

CMV status and terminally differentiated CD8⁺ Tc

Twenty of the 90 tested DS children are CMV⁺, they all have a latent infection (IgG⁺IgM⁺; PCR Avidity^{hi}). The median absolute number of CD45RA⁺CD27⁻ terminally differentiated Tc in CMV⁺ DS children is 0.079×10^9 cells/l (range 0.007-0.36), and 0.017×10^9 cells/l (range 0.0004-0.22) in CMV⁻ DS children ($p < 0.001$). In CMV⁺ healthy children a median absolute number of CD45RA⁺CD27⁻ terminally differentiated Tc of 0.067×10^9 cells/l is described [22]. Higher absolute numbers are described in children with primary CMV infection during immunosuppressive therapy (median 0.413×10^9 cells/l) and in CMV⁺ HIV-1 infected children (median 0.369×10^9 cells/l) [22, 23] (Figure 3).

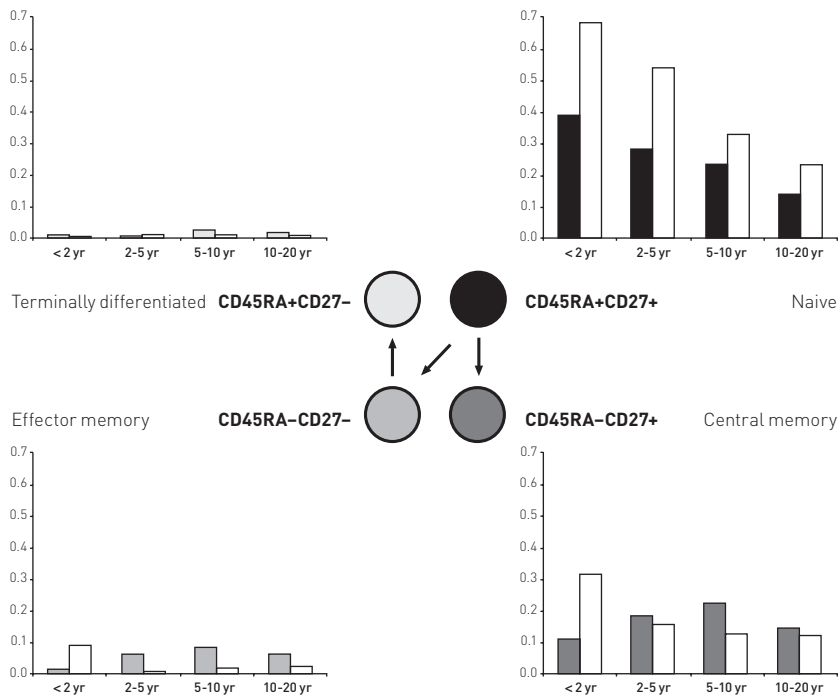


Figure 2 Median absolute numbers (x 10⁹/l) of cytotoxic T-lymphocyte subpopulations per age group.

Grey and black bars: Down syndrome children; white bars: corresponding age-matched reference values.

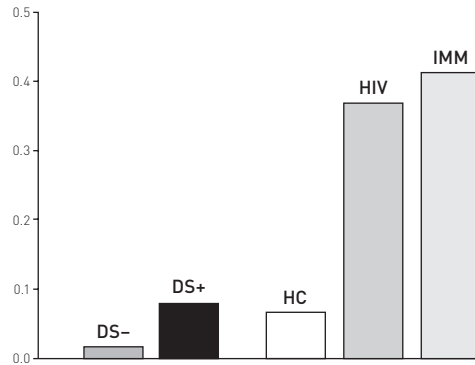


Figure 3 Median absolute numbers ($\times 10^9/l$) of terminally differentiated cytotoxic T-lymphocytes per group.

CMV: cytomegalovirus; DS: Down syndrome; HC: healthy children; HIV: human immunodeficiency virus infected; IMM: receiving immunosuppressive medication during primary cytomegalovirus infection.

DISCUSSION

DS children lack the expansion of both naive T_c and naive T_h normally seen in the first years of life; memory T_c and memory T_h are not influenced by age in either DS or AMC children. With advancing age, numbers of memory T_h, terminally differentiated T_c and TCR- $\gamma\delta^+$ T-lymphocytes normally increase [17]. Despite earlier statements in the literature suggesting precocious ageing of T-lymphocytes in DS, no early shift towards these T-lymphocyte subsets occurred in our DS cohort. A more likely explanation of the observed T-lymphocyte subset alterations in DS children would therefore be that the decreased numbers of T_c but particularly of T_h are the result of (partial) failure of T-lymphocyte generation, an intrinsic T-lymphocyte defect, increased apoptosis or a combination of these.

It is interesting to speculate about this finding. Apoptosis data in DS is scarce, but Elsayed [26] recently described increased early apoptosis markers in DS T-lymphocytes. Thymic alterations in DS are well-known [9, 10, 12, 13], and are already described in DS fetuses [27], suggesting T-lymphocyte generation is impaired by a defect in the DS thymus. This situation resembles children with DiGeorge syndrome who have a smaller or absent thymus; they demonstrate decreased (total) T_h and T_c subsets as well [28, 29]. In DiGeorge syndrome, however, most cases appear to gradually reach T-lymphocyte levels of healthy adults over time. In comparison, naive T_c in our DS children reach normal levels during adolescence, but naive T_h remain decreased. It is

still uncertain whether these cells function normally, having shown such a profound lack of the antigen-driven expansion in earlier years. In vitro tests of T-lymphocyte function support this hypothesis of impaired functioning [30].

Clinically speaking, DS children do not show signs of a profound T-lymphocyte deficiency. This corresponds with our finding that CMV⁺ DS children show absolute numbers of terminally differentiated T_c comparable to healthy children, not to immunocompromised, e.g. HIV⁺, children.

In conclusion, the observed T-lymphocyte alterations in DS are more likely caused by an intrinsic defect than by early senescence of the immune system. In the future, functional studies of T-lymphocytes may help to differentiate between a defect primarily originating in the thymus (as in DiGeorge syndrome), a defect in the T-lymphocytes themselves, increased apoptosis, or a combination of these options.

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