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Both Normal Memory Counts and Decreased Naive Cells Favor Intrinsic Defect Over Early Senescence of Down Syndrome T Lymphocytes

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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 aging. 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; this is more logically explainable with an intrinsic T lymphocyte defect. Furthermore, memory cell numbers are not different from age-matched controls (AMC), which does not support the hypothesis of precocious aging. Although the absolute numbers of T lymphocyte subpopulations approach AMC levels toward 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 with healthy children, not increased numbers as are seen in immunocompromised hosts. **(*Pediatr Res* 67: 557–562, 2010)**

Down syndrome (DS) is associated with a high frequency of hematological 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 aging 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 noninstitutionalized DS children (49 males; mean age, 7 y; 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.* preoperative 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 (control) 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 virus (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; IQ Products, Groningen, The Netherlands), CD8 (PE-Cy5; Immunotech), CD14 [PE; Becton Dickinson (BD), San Jose, CA], CD15 (FITC; IQ Products), CD16/CD56 (FITC; BD), CD19 (PE-Cy5; Immunotech), CD27 (FITC; BD), CD45 (PE-Cy5; Immunotech), CD45RA (PE; Coulter Immunology,

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Abbreviations: AMC, Age-matched control; **BD,** Becton Dickinson; **CMV,** Cytomegalovirus; **DS,** Down syndrome; **NK,** Natural killer; **PE,** Phycoerythrin; **PE-Cy5,** PE-cyanin 5; **Tc,** Cytotoxic T; **Th,** Helper T

Hialeah, FL), CD45RO (FITC; Serotec DPC, Apeldoorn, The Netherlands), TCR-$\alpha\beta$ (PE-Cy5;BD), and TCR-$\gamma\delta$ (PE; BD).

Aliquots were incubated for 15 min 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/or$CD56^+CD3^-$), 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) 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 ± 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); from 5 DS children, serum was not available. IgG avidity tests (VIDAS, Biomerieux) were performed to differentiate between recent (<3 mo) 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 y), 3) increased infection rate (age at inclusion >8 y), and 4) increased infection rate until, but not after the age of 8 y. 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 y) and group (DS and AMC). Age groups with low numbers of AMC children were excluded (<2; >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 are 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 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 (Fig. 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 (Fig. 2).

TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ $CD3^+$ T lymphocytes. The absolute number of TCR-$\gamma\delta$+ 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-$\alpha\beta$+ T lymphocytes follow the pattern of total T lymphocytes.

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) (Fig. 3).

DISCUSSION

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

It is interesting to speculate about this finding. Apoptosis data in DS is scarce, but Elsayed and Elsayed (26) recently described increased early apoptosis markers in DS T lymphocytes. Thymic alterations in DS are well-known (9,10,12,13),

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

	9–15 months	15–24 months	2–5 years	5–10 years	10–16 years	>16 years	DS vs AMC (2–16 years)*	Age effect (DS + AMC; 2–16 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) $n = 16$	1.39 (0.62–2.67) $n = 38$	1.00 (0.54–2.45) $n = 19$	1.25 (1.06–1.56) $n = 3$	$p = 0.003$	$p < 0.001$
	69 (58–81) $n = 11$	73 (66–81) $n = 8$	74 (65–84) $n = 16$	74 (58–89) $n = 38$	69 (59–85) $n = 19$	74 (72–81) $n = 3$	$p < 0.001$	NS
AMC	5.00 $n = 1$	2.30 $n = 1$	1.95 (1.20–2.90) $n = 10$	1.85 (1.50–2.40) $n = 8$	1.60 (0.80–2.40) $n = 11$	1.40 (1.10–1.70) $n = 2$		
	70 $n = 1$	60 $n = 1$	66 (59–71) $n = 10$	69 (58–72) $n = 8$	65 (59–77) $n = 11$	69 (63–75) $n = 2$		
Th								
DS	1.22 (0.69–2.97)	1.36 (0.70–1.59)	0.78 (0.04–1.67)	0.63 (0.30–1.40)	0.59 (0.28–1.16)	0.81 (0.48–0.92)	$p < 0.001$	$p < 0.001$
	69 (45–79)	62 (47–78)	55 (41–71)	46 (25–65)	58 (31–65)	52 (45–73)	$p < 0.001$	NS
AMC	3.30	1.40	1.15 (0.70–2.00)	1.00 (0.90–1.60)	0.80 (0.50–1.20)	0.95 (0.80–1.10)		
	65	60	60 (52–69)	65 (49–71)	56 (41–73)	70 (68–71)		
Tc								
DS	0.67 (0.20–1.20)	0.76 (0.38–1.32)	0.54 (0.28–1.41)	0.70 (0.27–1.45)	0.45 (0.18–1.15)	0.51 (0.33–0.78)	$p < 0.001$	NS
	30 (20–41)	36 (19–50)	41 (26–58)	47 (33–71)	40 (29–59)	48 (27–51)	$p < 0.001$	NS
AMC	1.40	0.80	0.70 (0.50–1.10)	0.50 (0.40–0.80)	0.50 (0.30–1.00)	0.40 (0.30–0.50)		
	29	33	35 (29–47)	29 (26–45)	36 (22–49)	29		
Th naive								
DS	0.91 (0.50–1.90)	0.90 (0.39–1.12)	0.49 (0.19–1.15)	0.28 (0.07–0.97)	0.20 (0.01–0.43)	0.19 (0.06–0.29)	$p < 0.001$	$p < 0.001$
	73 (63–83)	68 (55–72)	58 (37–70)	44 (11–69)	37 (2–59)	24 (7–60)	$p < 0.001$	$p < 0.001$
AMC	2.80	1.16	0.82 (0.41–1.60)	0.73 (0.64–1.30)	0.54 (0.27–0.80)	0.57 (0.44–0.70)		
	86	83	70 (58–80)	72 (64–80)	61 (54–67)	59 (55–64)		
Th memory								
DS	0.22 (0.13–0.72)	0.25 (0.16–0.35)	0.27 (0.14–0.39)	0.27 (0.12–0.51)	0.25 (0.15–0.65)	0.50 (0.15–0.66)	NS	NS
	18 (10–28)	23 (16–33)	32 (22–50)	45 (21–74)	52 (30–70)	62 (31–72)	$p < 0.001$	$p < 0.001$
AMC	0.36	0.17	0.29 (0.18–0.38)	0.24 (0.14–0.39)	0.25 (0.12–0.44)	0.31 (0.27–0.43)		
	11	12	23 (16–38)	23 (15–30)	31 (28–39)	32 (31–34)		
Tc naive								
DS	0.45 (0.11–0.83)	0.26 (0.14–0.63)	0.29 (0.09–0.85)	0.23 (0.03–0.62)	0.16 (0.05–0.55)	0.11 (0.08–0.15)	$p = 0.001$	$p < 0.001$
	72 (57–82)	53 (19–82)	47 (18–86)	42 (10–74)	41 (13–69)	31 (11–31)	$p < 0.001$	NS
AMC	0.64	0.69	0.53 (0.27–0.71)	0.34 (0.23–0.54)	0.25 (0.16–0.64)	0.23 (0.15–0.30)		
	46	86	66 (49–89)	68 (39–77)	57 (37–80)	54 (49–59)		
Tc centr mem								
DS	0.15 (0.04–0.34)	0.13 (0.06–0.26)	0.19 (0.06–0.91)	0.23 (0.09–0.65)	0.14 (0.07–0.50)	0.23 (0.20–0.25)	$p = 0.012$	NS
	23 (15–41)	23 (8–35)	39 (11–65)	37 (12–62)	37 (20–60)	50 (30–62)	$p = 0.001$	NS
AMC	0.53	0.10	0.16 (0.08–0.32)	0.13 (0.07–0.31)	0.12 (0.07–0.23)	0.13 (0.09–0.17)		
	38	12	28 (11–33)	26 (17–56)	32 (13–39)	32 (30–34)		
Tc eff mem								
DS	0.01 (0.00–0.02)	0.04 (0.00–0.54)	0.06 (0.01–0.61)	0.09 (0.01–0.61)	0.07 (0.01–0.68)	0.09 (0.03–0.70)	$p = 0.001$	NS
	1 (0–3)	9 (0–45)	4 (1–34)	8 (1–42)	7 (2–48)	10 (3–52)	$p = 0.008$	NS
AMC	0.17	0.01	0.01 (0.00–0.14)	0.02 (0.01–0.38)	0.02 (0.01–0.16)	0.03 (0.01–0.04)		
	12	1	2 (0–13)	4 (2–6)	7 (1–22)	9 (2–15)		

(*Continued*)

Table 1. (*Continued*)

	9–15 months	15–24 months	2–5 years	5–10 years	10–16 years	>16 years	DS vs AMC (2–16 years)*	Age effect (DS + AMC; 2–16 years)†
Tc term diff								
DS	0.01 (0.00–0.06)	0.02 (0.00–0.21)	0.01 (0.00–0.32)	0.03 (0.01–0.38)	0.02 (0.00–0.21)	0.04 (0.01–0.06)	NS	NS
	2 (0–9)	3 (0–18)	1 (0–31)	5 (1–41)	8 (1–27)	7 (3–8)	$p = 0.044$	NS
AMC	0.05	0.01	0.00 (0.00–0.11)	0.02 (0.00–0.06)	0.02 (0.00–0.09)	0.02 (0.02–0.03)		
	3	1	2 (0–10)	3 (1–10)	4 (2–10)	5 (5–5)		
$CD3^+TCR\alpha\beta^+$								
DS	1.89 (0.91–3.75)	1.94 (1.15–2.65)	1.44 (0.66–2.69)	1.20 (0.57–2.49)	0.91 (0.49–2.12)	1.19 (0.87–1.36)	$p = 0.001$	$p = 0.001$
	93 (77–98)	95 (90–96)	91 (68–97)	89 (61–95)	91 (74–98)	88 (82–96)	NS	NS
AMC	4.83	2.13	1.79 (1.07–2.76)	1.64 (1.38–2.28)	1.42 (0.74–2.17)	1.34 (1.05–1.63)		
	97	93	92 (86–97)	93 (84–98)	89 (80–96)	96 (96–96)		
$CD3^+TCR\gamma\delta^+$								
DS	0.17 (0.03–0.52)	0.11 (0.05–0.18)	0.13 (0.04–0.53)	0.14 (0.04–0.66)	0.09 (0.02–0.33)	0.18 (0.04–0.18)	$p = 0.043$	NS
	7 (2–23)	5 (3–10)	9 (3–32)	11 (5–39)	9 (2–26)	12 (3–17)	NS	NS
AMC	0.16	0.17	0.14 (0.07–0.33)	0.12 (0.03–3.24)	0.17 (0.06–1.48)	0.06 (0.05–0.06)		
	3	7	8 (3–14)	7 (2–16)	11 (4–19)	4 (4–5)		

Upper line: absolute numbers of T lymphocyte subpopulations (10^9 cells/L), lower line: relative numbers (%). The range is mentioned between brackets.

* Analysis of variance, effect of group (children aged 2–16 years).

† Analysis of variance, DS and AMC children together (2–16 years), effect of age.

NS, not significant; centr mem, central memory; eff mem, effector memory; term diff, terminally differentiated.

Table 2. *Clinical features*

	9–15 months ($n = 11$)	15–24 months ($n = 4$)	2–5 years ($n = 16$)	5–10 years ($n = 38$)	10–16 years ($n = 19$)	>16 years ($n = 3$)	Total ($n = 91$)
No increased infection rate (all ages)	$n = 5$		$n = 6$	$n = 5$	$n = 1$	$n = 2$	$n = 19$
Increased—mainly respiratory—infection rate (age at inclusion <8 y)	$n = 6$	$n = 4$	$n = 10$	$n = 20$			$n = 40$
Increased—mainly respiratory—infection rate until, but not after, the age of 8 y				$n = 6$	$n = 12$		$n = 18$
Increased—mainly respiratory—infection rate (age at inclusion >8 y)				$n = 7$	$n = 6$	$n = 1$	$n = 14$
Celiac disease	$n = 0$	$n = 1$	$n = 1$	$n = 2$	$n = 1$	$n = 0$	$n = 5$
Autoimmune hypothyroidism	$n = 0$	$n = 1$	$n = 0$	$n = 1$	$n = 1$	$n = 0$	$n = 3$

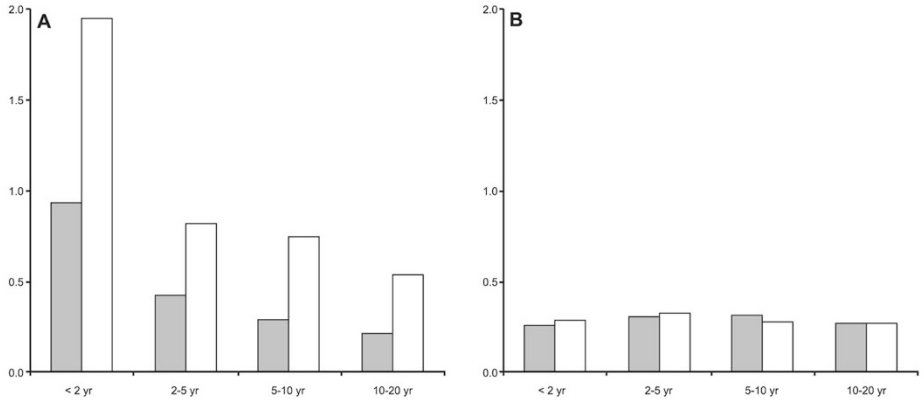

Figure 1. Median absolute numbers ($\times 10^9$/L) of helper T lymphocytes per age group. (*A*) Naive helper T lymphocytes ($CD3^+CD4^+CD45RA^+$); (*B*) memory helper T lymphocytes ($CD3^+CD4^+CD45RO^+$). *Gray bars*, DS children; *white bars*, age-matched reference values.

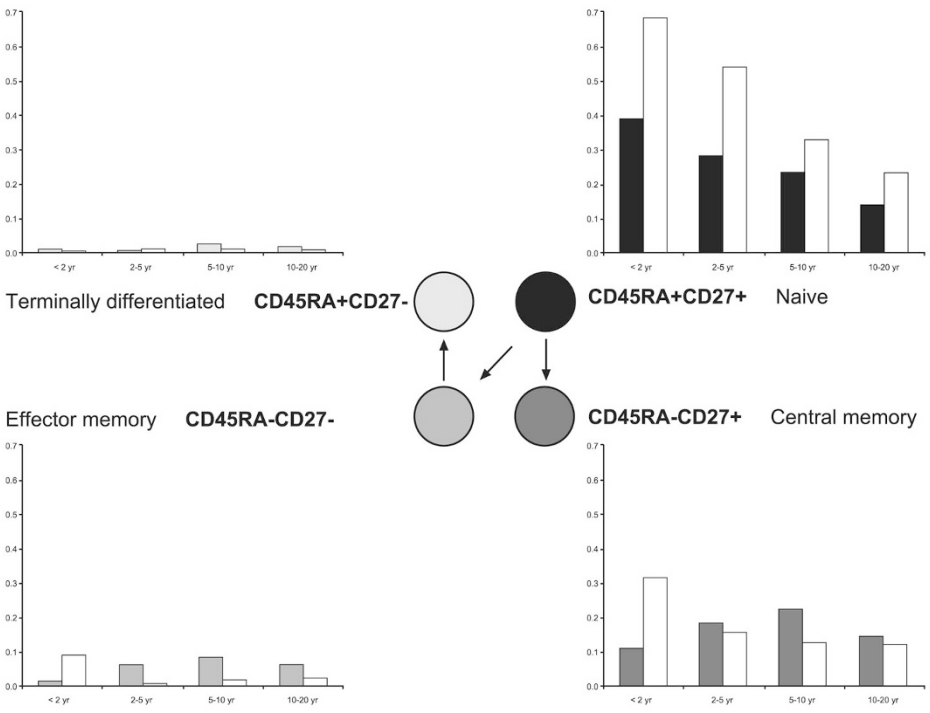

Figure 2. Median absolute numbers ($\times 10^9$/L) of cytotoxic T lymphocyte subpopulations per age group. *gray and black bars*, DS children; *white bars*, corresponding age-matched reference values.

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) Th and Tc 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 Tc in our DS children reach normal levels during adolescence, but naive Th 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 (30).

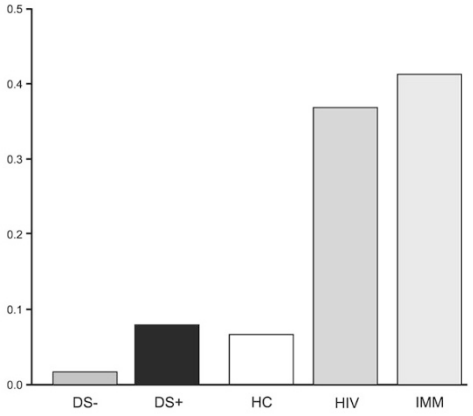

Figure 3. Median absolute numbers ($\times 10^9$/L) of terminally differentiated cytotoxic T lymphocytes per group. HC, healthy children and IMM, receiving immunosuppressive medication during primary cytomegalovirus infection.

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 Tc 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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