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Decreased Thymic Output Accounts for Decreased Naive T Cell Numbers in Children with Down Syndrome

Beatrijs L. P. Bloemers,* Louis Bont,* Roel A. de Weger,† Sigrid A. Otto,‡ Jose A. Borghans,‡ and Kiki Tesselaar‡

Children with Down syndrome (DS) have low numbers of naive T cells and abnormal thymus development and function. Because next to thymic production, peripheral proliferation greatly contributes to naive T cell generation in healthy children, we examined the cause of reduced naive T cell numbers in children with DS. Compared with aged matched controls, the total number of signal joint TCR excision circles (sjTREC) per ml blood was reduced in DS. Reduced frequencies and absolute numbers of protein tyrosine kinase 7-positive recent thymic emigrants, but similar levels of naive T cell apoptosis and Ag-driven activation in DS, suggested that reduced thymic output and not increased peripheral loss of naive T cells caused the reduced sjTREC numbers. We found no support for defective peripheral generation of naive T cells in DS. In DS the naive T cells responded to IL-7 and, based on Ki-67 expression, had similar proliferation rates as in healthy controls. sjTREC content per naive CD8+ T cells was not increased, but even decreased, pointing to increased survival or peripheral generation of naive T cells in DS. In conclusion, we show in this study that reduced thymic output, but not reduced peripheral generation nor increased loss of naive T cells, results in the low naive T cell numbers found in DS. *The Journal of Immunology, 2011, 186: 4500–4507.

The most common chromosomal abnormality of live-born infants is Down syndrome (DS). Children with DS have a high morbidity because of respiratory tract infections (1, 2). In addition, various immunological impairments are associated with DS. Children with DS have a high incidence of hematologic malignancies and autoimmune diseases like hypothyroidism, celiac disease, and diabetes mellitus (3–7).

Studies on the adaptive immune system of DS have shown that total lymphocyte numbers are decreased in this population (8–14). This holds true for the CD4+ and CD8+ T cell subsets, especially in the first 2 y of life, but becomes less pronounced with increasing age and eventually normalizes around the age of 16 y. Subdivision of T cells into naive and memory cells showed that in fact only the naive subsets are smaller compared with healthy controls (8, 9, 11–13). In contrast, the fraction of CD8+ T cells within the T cell compartment is increased, and the fraction of CD4+ T cells decreased (9). Percentages of naive cells have been described to be decreased and memory and effector subsets to be increased in both CD4+ and CD8+ T cells (15–17). Several studies have ascribed the immunologic impairment of T cells in DS to abnormal thymus development and function (16, 18–21). Children with DS show increased cortical thymocyte depletion, cystic changes, and fibrosis, suggestive of an accelerated involution of the thymus as seen in elderly. Although thymocyte maturation appears disturbed, normal mature T cells expressing CD3 and TCRαβ are found in peripheral blood (21, 22).

Establishment and maintenance of the peripheral naive T cell compartment are dynamic processes. T cells are produced by the thymus and are released in peripheral blood as recent thymic emigrants (RTE). Because these cells are the most proximal to the thymus, they are essential for maintaining a diverse αβ TCR repertoire. This proximity is also reflected by their high content of signal joint TCR gene excision circles (sjTRECs), which are circular DNA products of intrathymic V(D)J recombination. Peripheral proliferation of naive T cells and longevity are the other mechanisms contributing to establishment and maintenance of the naive T cell pool. Main drivers of these processes are IL-7 (23) and TCR-MHC/self-peptide ligand interactions (24, 25). Based on phenotypic markers, several naive CD4+ T cell subsets with different dynamic histories can be distinguished (24). Recently, protein tyrosine kinase 7 (PTK7) has been described as a novel marker for CD4+ RTE (26). Naive CD31-positive and PTK7-positive CD4+ T cells are considered to be the subset most proximal to the thymus, and MHC/self-peptide ligand-induced proliferation of these cells has been suggested to lead to loss of PTK7 and subsequently CD31 expression. IL-7 preferentially induces proliferation of the CD31-positive subset, thereby downmodulating CD127 expression, but not inducing any other phenotypical changes (27).

Roat et al. (17, 28) showed that children with DS have lower percentages of sjTREC-positive cells in PBMCs compared with controls. They suggested that this was the result of thymic impairment. Although this conclusion could explain their observation, sjTREC dynamics and T cell homeostasis are also influenced by cellular life span and division (29). In the current study, we addressed naive T cell dynamics in children with DS now incorporating thymic output, T cell proliferation, and T cell loss by Ag-driven differentiation or apoptosis. From our combined results, we conclude that decreased thymic output is responsible for the low naive T cell numbers in children with DS.
Materials and Methods

Study population

Whole blood samples were obtained by venupuncture from healthy children with DS and controls. Forty-seven children with DS who attended the outpatient clinic of the Wilhelmina Children’s Hospital Utrecht, The Netherlands, were included in six age groups from 0.1 to 12 y. Because the largest developmental changes in the immune system are described in approximately the first 2 y of life, three times more children were included in the age group of 0–2 y (n = 16) compared with the other age groups, in which five children per 2-y stratum were included (30, 31). Children with DS and a history of surgery in the previous 2 mo were excluded. An equal number of age-matched, otherwise healthy children, who were admitted to the hospital for elective urologic, plastic, ophthalmologic, or general surgery, were included as controls. To minimize interference on immunologic parameters, blood was drawn prior to or directly after anesthesia was given. None of the children showed signs of acute infection at the time that blood was drawn. Five children with DS and eight control children used prophylactic antibiotics at the time of sampling. Four to 10 ml blood was drawn from each individual. In four children with DS and three controls, the absolute lymphocyte count could not be determined. PBMCs could not be isolated in all three children of both groups. FACS staining could not be performed in four children with DS and six healthy controls and only partially in the remaining children. For all cases parental written informed consent was obtained. The research protocol was approved by the medical ethics committee of the University Medical Centre Utrecht.

Cell preparation and cultures

PBMCs were isolated from heparinized blood samples, and plasma was isolated from EDTA-anticoagulated blood samples. PBMCs were obtained by Ficoll-Paque density-gradient centrifugation and stored in liquid nitrogen until further processing. CD4+ and CD8+ T cells were purified from thawed PBMCs by magnetic bead separation using the MiniMACS mini-tis kits, according to manufacturer’s instructions (Miltenyi Biotec). To measure sjTREC content within naive (CD27+CD45RO−) and memory (CD27−CD45RO+) CD4+ and CD8+ T cells, these subsets were isolated by cell sorting on a FACSAria (BD Biosciences). PBMCs were cultured with or without IL-7 at a final concentration of 10 ng/ml IL-7 (Sigma-Aldrich) in RPMI 1640/10% FCS culture medium for 7 d. For the qTREC assay, cryopreserved PBMCs were thawed and cells were either stained directly with the apoptotic marker annexin V (BD Biosciences) or after overnight culture in medium.

Flow cytometry

Thawed cryopreserved PBMCs were used for characterization of the T cell compartment. PBMCs were stained with mAbs to CD3-Pacific Blue, CD4-Pacific Blue, CD45RO-PerCP-Cy5.5, CD44-allophycocyanin-Cy7, CD8-PerCP-Cy5.5, CD45RO-PE-Cy7, CD8-PE, CD27-PerCP-Cy5.5, CD27-allophycocyanin-Cy7, CD8-APC, CD28-PerCP-Cy5.5, CD25-PE-Cy7, CD127-FITC, and CD38-PE-Cy7 (BD Biosciences). Intracellular staining was performed to measure expression of Ki67 (DakoCytomation). In short, PBMCs were stained, fixed, and permeabilized with Cytofix/Cytoperm (BD Biosciences) and washed twice with permeabilization wash buffer. Next, cells were stained with Ab directed against Ki67, washed twice with permeabilization buffer, and resuspended in FACS buffer. Affinity-purified rabbit IgG anti-mouse PTK7 was provided by X. Lu (Department of Cell Biology, University of Virginia, Charlottesville, VA). For surface PTK7 staining, Alexa Fluor 488-conjugated goat anti-rabbit IgG was used as a secondary Ab. As a control for specific binding, cells were stained with an identical mixture without the anti-mouse PTK7 Ab. Annexin V staining was used according to manufacturer’s protocol to determine the fraction of apoptotic cells. In short, after surface staining of T cell subsets, cells were washed, incubated with annexin V Ab for 15 min, and washed again. Cells were resuspended in annexin V buffer and analyzed. Forward/Sideward scatter was used to exclude dead cells. Cellular fluorescence was measured using a LSRII flow cytometer (BD Biosciences) and analyzed with FACS Diva software (BD Biosciences). For each sample, minimally 50,000 events were present in the lymphocyte gate (see Supplemental Fig. 1 for gate definition). FACS results were only included in the analysis if a specific subpopulation >25 events were counted.

Population definitions

CD8+CD3+ T cells were subdivided into naive (CD27−CD45RO−), memory (CD27+CD45RO−), memory effector (CD27−CD45RO+), and effector (CD27−CD45RO+) subsets (32). In analogy, we have used the same definition of naive and Ag-experienced subsets for CD4+ T cells. In addition, in a limited number of patients, expression of CD25, CD31, CD38, HLA-DR, CD127, KI67, and PTK7 within naive and memory subsets was determined. Examples of the gating strategy used are shown in Supplemental Fig. 1. Absolute lymphocyte count was determined using automated Multi Angle Polarized Scatter Separation plus three-color fluorescent on a CELL-DYN Sapphire (Abbott Diagnostics) and was used to calculate absolute numbers of the indicated lymphocyte subsets by multiplying the percentage of the subset as obtained by flow cytometry with the absolute lymphocyte number.

sjTREC analysis

sjTREC numbers were determined by real-time PCR on genomic DNA of CD4+ and CD8+ T cells, as described previously (29, 33). DNA was purified using the Nucleospin Blood QuickPure, according to manufacturer’s instructions (Macherey-Nagel). sjTREC content per CD4+ or CD8+ T cell was calculated by dividing the sjTREC content/μg DNA by 150,000 (assuming that 1 μg DNA correlates with 150,000 T cells). Total numbers of sjTRECs were calculated as the sjTREC content per CD4+ or CD8+ T cell multiplied by the absolute CD4+ or CD8+ T cell count/ml.

IL-7 ELISA

Plasma samples were frozen 3–12 h after they were obtained. A short period of IL-7 might influence the results of our tests using samples with a wide time frame before freezing. To exclude time as a possible dependent factor in the results of our test, we examined the influence of time until freezing of the sample on IL-7 plasma levels in three healthy donors. No significant differences were found in IL-7 plasma levels of samples frozen between 3 and 12 h after sampling (data not shown). None of the samples had been thawed previously. After thawing, samples were analyzed using an ELISA (Quantikine HS; R&D Systems), according to the manufacturer’s recommendations. All samples were run in duplicate. A standard curve was prepared by serial dilutions.

Statistical analysis

Differences in results between children with DS and healthy controls were compared using χ2 test, Student t test, or nonparametric tests in case of unequal distribution between the groups. Values are expressed as the means ± SEM. In all analysis, regression analysis, including sex and age, was performed to evaluate whether differences existed between children with DS and healthy controls. All statistical analyses were performed using the software program SPSS for Windows (version 12.0.2; SPSS, Chicago, IL). A p value ≤0.05 was considered significant. A sensitivity analysis was performed to determine whether a history of corrective surgery for congenital heart disease in children with DS could have influenced the outcome of the results of this study. All analyses were repeated for children without corrective heart surgery.

Results

Forty-seven children with DS, ages 0–12 y, were included in the study. An equal number of healthy age-matched children admitted to the hospital for elective surgery were included as controls. Children were divided in eight age strata of 2 y with five to six children each. We used narrower strata in the first 2 y of life because the largest immunological changes are found during those years. Because of restrictions in blood volume that could be drawn and concomitant yield of PBMCs, some experiments could not be performed in all 47 children (for details, see Materials and Methods). In our study group of children with DS, 20 children with DS had congenital heart disease in some degree. Six of them had no corrective surgery. In four children, only analysis of IL-7 levels has been performed. Ten of 14 children, who had corrective heart surgery, had an age >6.5 y when blood was drawn.

Decreased absolute numbers of naive CD4+ and CD8+ T cells

We confirmed that children with DS have lower absolute numbers of naive CD4+ and CD8+ T cells compared with healthy controls (0.91 versus 1.84 × 109/l naive CD4+, p < 0.001 and 0.41 versus 0.83 × 109/l naive CD8+, p < 0.001) (Fig. 1) (8–13). This effect was seen at all ages, although differences between groups became smaller with increasing age. Absolute numbers of naive T cells were negatively correlated with age in both groups. Normal ab-
Solute numbers of memory and effector subsets of CD4+ and CD8+ T cells were found in children with DS compared with controls (Supplemental Fig. 2A, 2B).

Decreased sjTRECs and RTE numbers in children with DS
Several studies have shown thymic abnormalities in children with DS. To assess the cause of low absolute numbers of naive T cells in children with DS, we first measured total sjTREC numbers/ml blood. Both for CD4+ and CD8+ T cells we found total sjTREC numbers to be >2-fold decreased in children with DS compared with controls (mean 0.23 versus 0.50 × 10^9/l, p = 0.003 and 0.07 versus 0.13 × 10^9/l, p = 0.001, respectively; Fig. 2A, 2B). Regression analysis of total sjTREC numbers in CD4+ and CD8+ T cells showed independent effects of DS and age.

Next, we measured the absolute number and percentage of CD4+ RTEs as determined by the expression of PTK7 on naive CD4+ T cells. In children with DS, the absolute number and the percentage of PTK7-positive cells within naive CD4+ T cells were decreased (0.10 versus 0.32 × 10^9/l, p < 0.001 and 10.2 versus 18.6%, p = 0.008, respectively; Fig. 2C, 2D). Regression analysis of both absolute numbers and percentages of PTK7-positive cells within naive CD4+ T cells showed independent effects of DS and age.

FIGURE 1. Children with DS have decreased absolute naive T cell counts. Age distribution of absolute numbers of naive CD4+ (A) and naive CD8+ (B) T cell subsets in children with DS (n = 36) compared with healthy controls (HC) (n = 35). Statistical analysis is shown for the difference between the group of children with DS and the group of healthy controls. Regression analysis showed independent effects of DS and age.

FIGURE 2. Children with DS have lower TREC numbers and RTE numbers and frequency compared with healthy controls (HC). Age distribution of CD4+ (A) and CD8+ (B) TREC counts/l blood, and absolute numbers (C) and percentages (D) of PTK7+ RTE within naive CD4+ T cells in children with DS (n = 30, n = 30, n = 24, and n = 27, respectively) compared with healthy controls (n = 32, n = 32, n = 23, and n = 26, respectively). Statistical analysis is shown for the difference between the group of children with DS and the group of healthy controls. Regression analysis showed independent effects of DS and age.
No increased loss of peripheral naive T cells in DS

To determine whether reduced thymic output or increased loss causes the observed reduction in sjTREC numbers and RTE, we measured the level of peripheral naive T cell loss by flow cytometry. We analyzed the percentage of naive CD4+ and CD8+ T cells expressing the apoptosis marker annexin V. Annexin expression was analyzed ex vivo and after 16 h of culture in the presence or absence of polyclonal stimulation with anti-CD3 mAb. Under all circumstances there was no difference between the fraction of apoptotic naive CD4+ and CD8+ T cells between children with DS and healthy controls (Fig. 3A, 3B).

Next, we analyzed the level of Ag-induced activation. We reasoned that, as seen in HIV infection, increased Ag-induced activation could lead to naive T cell loss. The children with DS in this study did not show any clinical signs of acute or chronic infection. We used expression of CD25 in naive T cells as a measure of recent antigenic activation of CD8+ T cells (34) (our personal observation). No differences were found in the absolute number of recently activated (CD25+CD45RO−CD27+) CD8+ T cells (Fig. 3C). Analysis of T cell activation using the activation markers CD38+ and HLA-DR+ gave also no indications for increased T cell activation, as the absolute numbers of CD38+HLA-DR+ CD4+ and CD8+ T cells were similar in DS children compared with healthy controls (Supplemental Fig. 3A, 3B). Because we found no indications of increased peripheral naive CD4+ or CD8+ T cell loss, we concluded that in children with DS the reduction in sjTREC numbers and RTE resulted from decreased thymic output.

No indication for reduced peripheral generation of naive T cells

Besides thymic output, peripheral proliferation contributes largely to the establishment of the naive T cell pool (35–37). We assessed possible disturbances in peripheral proliferation using the proliferation marker Ki67. Normal absolute numbers (Supplemental Fig. 4A, 4B) and percentages of naive CD4+ T cells and naive CD8+ T cells expressing Ki67 were found in children with DS compared with controls (Fig. 4A, 4B). For naive CD4+ T cells, CD31 is a thymic proximity marker, and loss of CD31 expression has been suggested to reflect self Ag-driven proliferation (24, 25, 27). In line with the Ki-67 data, analysis of the fraction of cells not expressing CD31 among naive CD4+ T cells was similar compared with healthy controls (33.3 versus 28.7%, p = 0.09; Fig. 4C). Thus, both analyses showed no evidence for reduced peripheral proliferation as a cause for the reduced naive T cell numbers in DS.

No indication for defective IL-7–dependent peripheral proliferation or survival

IL-7 is a cytokine important for T cell survival and proliferation, especially of naive T cells (23). Its production is regulated by an IL-7R–mediated feedback loop, which together with the utilization by T cells determines the serum and tissue levels of the cytokine. In human lymphopenic settings, IL-7 plasma levels are increased and are thought to increase low-affinity TCR-induced proliferation and enhance survival of naive T cells (38–40). We investigated whether IL-7 influenced peripheral naive T cell numbers in DS. In children with DS, significantly higher IL-7 plasma levels were found, which was not affected by age (7.5 versus 5.8 pg/ml, p = 0.03; Fig. 5A). However, no correlation between IL-7 plasma level and number of total (Supplemental Fig. 5A, 5B) and naive CD4+ or CD8+ T cells was found (Supplemental Fig. 5C, 5D). In vitro stimulation with IL-7 showed a normal capacity to respond to IL-7 in children with DS, as down-modulation of CD127, the limiting component of the IL-7R, was...
similar (data not shown). Ex vivo analysis of CD127 expression on naive T cells showed small increases in relative fraction of CD1272 within naive CD4+ (Fig. 5B) and CD8+ (Fig. 5C) subsets (24.6 versus 18.9%, p = 0.05 and 23.6 versus 12.0%, p < 0.001, respectively), suggestive of increased per cell IL-7 utilization and increased survival or proliferation. Regression analysis showed independent effects of age and DS in the CD4 subset and no effect of age in the CD8 subset. Thus, despite increased IL-7 plasma levels, we found no indications for a role of IL-7 in decreasing peripheral proliferation in children with DS.

**Cumulative effects over time: sjTREC content analysis**

To corroborate our conclusion that reduction in peripheral proliferation and increase of naive T cell loss are not the cause of reduced naive T cell numbers in children with DS, we measured sjTREC content of the CD4+ and CD8+ T cell compartment (Supplemental Fig. 6). In contrast to sjTREC numbers/ml blood, which reflect the balance between thymic production and T cell loss, sjTREC content per CD4+ or CD8+ T cell reflects the balance between thymic output, T cell proliferation, and loss, and small changes in proliferation or survival rates will accumulate over time in larger differences in the sjTREC content. When peripheral proliferation or survival per cell is decreased, the sjTREC content per T cell will become relatively higher. In agreement with our hypothesis, no significant changes were found in the sjTREC content of sorted naive CD4+ T cells in children with DS compared with healthy controls (median 0.15 versus 0.31, p = 0.08; Fig. 6A). For sorted naive CD8+ T cells (Fig. 6B), decreased sjTREC content was found in children with DS compared with healthy controls (median 0.17 versus 0.28, p = 0.04). Regression analysis showed independent effects of age and DS. The decreased sjTREC content in sorted naive CD8+ T cells is also in line with our hypothesis and even suggests increased proliferation or decreased loss in children with DS.

We performed a sensitivity analysis in which all analyses of this study were repeated, excluding children with a history of corrective heart surgery, to analyze the possible contribution of perioperative thymectomy in those children. All conclusions remained identical without loss of statistical significance for any analysis.

**Discussion**

Histopathologic studies of thymic tissue in DS have shown increased involution of the thymus and altered patterns of maturation of thymocytes (19, 21, 22). In addition, several studies have described low numbers of T cells, decreased cytokine production upon antigenic stimulation, and diminished Ab-dependent T cell-mediated cytotoxicity in DS (10, 13, 41). It was suggested that thymic insufficiency causes peripheral T cell dysfunction in DS and that decreased thymic output is a causative factor for the low numbers of naive T cells in these children. sjTREC analysis is the most applicable tool to determine thymic output in humans, but the results should be interpreted with care (29). Reduced sjTREC contents might be indicative of reduced thymic output, but are also influenced by peripheral mechanism of T cell maintenance. Because sjTRECs are circles of DNA spliced off during TCR rearrangement that do not replicate during mitosis, they are diluted upon cell division. In addition, longevity of naive T cells influences sjTREC content of cells by decreasing sjTREC content when naive T cells tend to live longer. For this reason, absolute numbers of sjTRECs per milliliter blood are used to provide information on thymic output, whereas the average sjTREC content per cell is used for determination of the replicative history. The combined results of our detailed study of T cell dynamics now put forward that in children with DS decreased thymic output is solely...
responsible for the decreased naive T cell numbers, and that peripheral mechanisms of naive T cell maintenance are fully functional in children with DS. In fact, based on the reduced sjTREC content in naive CD8+ T cells, we conclude that peripheral mechanisms counteract the reduced thymic output. Future studies on thymopoiesis, including analysis of thymic tissue, in particular of T cell subsets, their reactivity to IL-7, intrathymic precursor T cell proliferation, and the implication of growth hormones in thymopoiesis, might clarify the cause of decreased thymic output in children with DS.

To our knowledge, we are the first to report on RTEs identified by PTK7 in a large pediatric population after its description by Haines et al. (26) Within this healthy pediatric cohort, percentages of RTE were similar to percentages described by Haines. Regression analysis showed an age-dependent decline in the absolute numbers, but not frequency of PTK7-positive cells within naive CD4+ T cells in the blood between the age of 0 and 13 y. This age-independent frequency of RTE implies that no RTE-specific turnover changes take place with age, and therefore, that RTE and resident naive T cells are mostly regulated by the same mechanisms.

FIGURE 5. Children with DS have normal IL-7–dependent peripheral proliferation and survival. Plasma levels of IL-7 (A) measured by ELISA in children with DS (n = 29) compared with healthy controls (HC) (n = 35) at different ages. Regression analysis showed no effect of age. Flow cytometric analysis of relative numbers of CD127+ naive CD4+ (B) and CD8+ (C) T cells within the respective naive T cell pools in children with DS (n = 36 and n = 30, respectively) and healthy controls (n = 37 and n = 34, respectively). Statistical analysis is shown for the difference between the group of children with DS and the group of healthy controls. Regression analysis showed independent effects of age and DS in the CD4+ subset and no effect of age in the CD8+ subset.

FIGURE 6. Children with DS have normal or even decreased TREC content compared with healthy controls (HC). To determine whether lower TREC counts in CD4+ and CD8+ T cells reflect lower fraction of naive T cells within children with DS, TREC contents of purified naive CD4+ (A) and CD8+ (B) T cells were measured in children with DS (n = 9) and healthy controls (n = 9). Statistical analysis is shown for the difference between the group of children with DS and the group of healthy controls. Regression analysis showed independent effects of age and DS.
In previous DS literature, accelerated thymic involution and clinical findings, such as occurrence of Alzheimer’s disease at the young age of 40–50 y, have been ascribed to early senescence. Ageing of the immune system is difficult to define, but decline in naive T cell numbers, oligoclonal expansion of memory T cells starting around the sixth decade of life, and loss of TCR diversity are important aspects (42). Although the decreased numbers of naive T cells support the concept of accelerated ageing, other results are in apparent contrast. First, in accelerated ageing one would expect that absolute naive T cell numbers and total sjTREC counts are lower and keep declining over age at a higher rate than in controls. In children with DS, a higher rate of decline was only found in the first 2–4 y. Secondly, with ageing, naive and memory CD8+ T cells are lost, whereas CD8+ effector T cells increase (43, 44). In children with DS, over age no significant changes in absolute or relative numbers of CD8+ memory and effector T cells were shown. A third aspect of ageing is loss of TCR diversity caused by oligoclonal expansion, mostly within memory CD8+ T cells. When we used Vβ spectratyping of PBMCs to compare oligoclonality (45, 46) of children with DS and healthy controls, a larger number of TCR Vβ families that were oligoclonal were seen in children with DS (our unpublished results). Because TCR diversity was not measured within CD4+ and CD8+ T cell subsets, it is unclear whether this loss of TCR diversity found in children with DS reflects a relative increase of memory T cells or clonal expansion associated with homeostatic maintenance of the naive T cell compartment or a combination of both. If loss of TCR diversity in children with DS is not explained by early senescence, it might still play a role in the higher susceptibility to infections in children with DS. Future studies are needed to explore TCR diversity and functional loss of the immune system at different ages to further clarify the concept of ageing in patients with DS. Future longitudinal studies that correlate these immunologic parameters to clinical data could give better insight in the role of decreased naive T cells in the morbidity found in children with DS.

The natural variance in values of the dynamic parameters, even in a healthy population, can be quite substantial (~4-fold, up to 10-fold for TREC content). Next to genetic disposition, factors like age and infection status are known effectors. Recent or chronic infections were exclusion criteria for our study, to prevent the effect of infection status. Despite our relative small group sizes and the large variance, we were able to find significant age effects and differences between DS and healthy controls. More important, for all results that were significantly different between DS and healthy controls, regression analysis showed an effect of DS that was independent of age. Based on the difference between DS and healthy controls, regression analysis showed an effect of DS that was independent of age. Based on the difference between DS and healthy controls, regression analysis showed an effect of DS that was independent of age.

In conclusion, to our knowledge, this is the first study that formally shows decreased thymic output as a cause of the diminished naive T cell pool in children with DS, confirming the hypothesis of thymic insufficiency. Evidence of an intrinsic naive T cell defect contributing to a diminished naive T cell pool in these children could not be provided. Our data suggested that IL-7–driven peripheral mechanisms might counteract the reduced thymic output. Future functional studies are needed to determine whether increased peripheral proliferation and reduced TCR diversity of naive T cells have a role in the morbidity described in DS.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental figure 1. Characterization of T-cell subsets. (A) Representative examples of the gating strategy used for the measurements of the different T-cell subsets and the marker expression within these subset as described in the Materials and Methods section for a child with DS (upper plots) and a healthy control (lower plots). Lymphocytes were defined based on their forward and side scatter (P1, plot 1) and CD3⁺ T-cells (P2, plot 2) were gated within this lymphocyte gate. CD4⁺ (P3, plot 3) and CD8⁺ (P11, plot 6) T-cells were defined as CD4⁺ and CD8⁺ cells within the CD3⁺ lymphocyte gate. Within the CD4⁺ (P3) and CD8⁺ T-cells (P11) naive CD27⁺CD45R0⁻ (P4 respectively P12, in plot 4 and 7), memory CD27⁺CD45R0⁺ (P5 respectively P13, in plot 4 and 7), memory effector CD27⁺CD45R0⁺ (P6 respectively P14, in plot 4 and 7) and effector CD27⁺CD45R0⁻ (P7 respectively P15, in plot 4 and 7) subsets are shown. CD31 expression within naive CD27⁺CD45R0⁻CD4⁺ T-cells (P4) is shown in plot 5. (B) A representative example of the gating strategy for the determination of the fraction of PTK7 expressing cells within CD27⁺CD45R0⁻CD4⁺ T-cells (P4) is shown (plot 8) with the isotype control (plot 9) in a child with DS. (C) A representative example of the gating strategy for the determination of the percentage of Ki67 expressing cells within naive CD27⁺CD45R0⁻CD4⁺ (plot 10) and CD8⁺ (plot 11) T-cell subsets is shown in a child with DS. (D) A representative example of a second gating strategy to visualize the cut off for Ki67 positive naive CD27⁺CD45R0⁻CD4⁺ (plot 12) and CD8⁺ (plot 13) T-cells.

Supplemental figure 2. Children with DS have normal numbers of memory and effector T-cells. Pooled data of absolute numbers of CD4⁺ (A) and CD8⁺ (B) T-cell subsets in children with DS (n=36) compared to HC (n=35) (mean ± SEM). M
indicates memory CD27^CD45R0^, EM memory effector CD27^CD45R0^ and E indicates effector CD27^CD45R0^ subsets.

*Significant difference (P < .001)

**Supplemental figure 3. Children with DS have normal activation of naive CD4^+ and CD8^+ T-cells.** The absolute number of CD38^+ HLA-DR^+ CD4^+ (A) and CD8^+ (B) T-cells was determined by flow cytometry in children with DS (n=33) and HC (n=35).

**Supplemental figure 4. Children with DS have normal peripheral generation of naive CD4^+ and CD8^+ T-cell subsets.** Flow cytometric analysis of absolute numbers of Ki67^+ naive CD4^+ (A) and CD8^+ (B) T-cells in children with DS (n=33) and HC (n=34) at different ages.

**Supplemental figure 5. Children with DS show no correlation of IL-7 plasma levels and absolute T-cell numbers.** Correlation between IL-7 plasma levels and absolute numbers of total and naive CD4^+ (A, C) and CD8^+ (B, D) T-cells in children with DS (n= 26) compared to HC (n=31).

**Supplemental figure 6. Children with DS have normal or even decreased TREC content compared to HC.** Age distribution of TRECs per CD4^+ (A) and CD8^+ (B) T-cell in children with DS (n=32) compared to HC (n=35) at different ages. Statistical analysis is shown for the difference between the group of children with DS and the group of healthy controls.