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Neonatal thymectomy reveals differentiation and plasticity within human naive T cells

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Introduction

After egress from the thymus, naive T cells continuously recirculate through the peripheral lymphoid tissues, surveying for their cognate antigen presented by dendritic cells. After priming, naive T cells differentiate into effector/memory T cells that can be divided into subsets based on their phenotype and function (1). The CD4+CD45RA+CCR7+ naive T cell compartment has long been regarded as merely a source for the effector/memory T cell population without a specific effector function. Naive CD4+ T cells indeed lack substantial production of effector cytokines that define the different T helper subset signatures. However, it was recently shown that human naive CD4+ T cells have the unique capability of producing large amounts of IL-8 (CXCL8), indicating that these cells do have a specific effector function, at least in newborns (2). How this functional signature relates to naive T cell dynamics and aging is unknown.

The thymus plays a central role in the generation and maintenance of naive T cells early in life. After the first year of life, naive T cell production in the thymus starts to decline, resulting in a very minimal production rate in healthy adults (3–6). Even though the adult thymus is still able to produce new naive T cells (7, 8), we and others have shown that, in contrast to what is seen in mice, most of the naive T cell population in humans is maintained by homeostatic proliferation (9, 10). Due to increased homeostatic proliferation or decreased cell death, only a marginal decrease of the overall size of the naive T cell pool is observed during aging (11–15). Interestingly, new insights indicate that there are also temporal dynamics within the naive CD4+ T cell pool. Differential expression of CD31 on naive CD4+ T cells seems to distinguish between a CD31+ subset enriched in recent thymic emigrants (RTEs) and a CD31− naive subset that has most likely arisen after homeostatic proliferation of CD31− naive CD4+ T cells (9, 16, 17). Indeed, the percentage of CD31− cells in the naive CD4+ T cell compartment decreases, while the percentage of CD31− naive CD4+ T cells increases during healthy aging (16–18). Although it is clear that both the thymus and homeostatic proliferation contribute to naive T cell homeostasis and that their relative contributions change over time, it remains unknown what the functional consequences are of these changes in naive T cell dynamics. Functional changes within the naive T cell compartment may have major implications for vaccination and immune intervention strategies, especially in the very young and elderly.

A unique model for studying human naive T cell dynamics and function is a group of patients that have undergone neonatal thymectomy during cardiac surgery. In these children, the proportion and number of both CD4+ and CD8+ T lymphocytes are significantly reduced, mainly affecting the naive T cell population, which is not seen after a thymectomy later in life (19–23). Furthermore, following neonatal thymectomy, the T cell compartment composition and phenotype resemble those of an adult, which has been regarded as premature immune aging (20). Interestingly, 5 to 10 years after neonatal thymectomy, many of the thymectomized (Tx) individuals show signs of thymic tissue regrowth and restoration of T cell numbers and T cell receptor excision circle (TREC) content, indicating an unexpected level of thymic tissue regenerative capacity (25, 24). It remains unknown, however, whether this thymic tissue regeneration is functional and able to restore the affected naive T cell compartment and reverse changes in composition.

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Results

Neonatal thymectomy results in significantly reduced naive CD4+ T cell percentages and numbers with a shift toward CD31+ within the naive compartment in the first years of life. In line with our previous observations (23), we observed a significant decrease in absolute CD3+, CD4+, and CD8+ T cell numbers in the first years after neonatal thymectomy (treatment 1–5 yr) compared with those of healthy controls (HCs) (Figure 1A). Within the CD4+ T cell compartment, the proportion of CD4+CD45RA+CCR7+ naive T cells was significantly reduced after neonatal thymectomy. Furthermore, our data provide further support for the strong regenerative capacity of the thymus, leading to functional restoration of the naive T cell compartment after neonatal thymectomy. These data provide what we believe to be new insights in the naive T cell compartment that change the current concept for the identification of truly naive T cells and RTEs.
Thymic tissue regeneration occurs in the majority of children and results in restoration of naive CD4+ T cell thymic output later in life. A decade or more after neonatal thymectomy (Tx > 10 yr), absolute CD4+ and CD8+ T cell numbers were no longer different from those of age-matched HCs (ref. 23 and Figure 2A). However, we observed a subgroup of Tx patients (n = 7) with a very low percentage of CD31-expressing CD45RA+CCR7+ naive CD4+ T cells in comparison with age-matched HCs (Figure 2B). Interestingly, a relative increase in the recently described (25) self-renewing stem cell memory T cells (Tscm) was noted after neonatal thymectomy (Figure 1D), although their absolute numbers were still lower compared with HCs (Supplemental Figure 1A). Overall, neonatal thymectomy profoundly affected the naive T cell compartment in numbers and composition, while absolute numbers of memory T cells were comparable in Tx and HC groups.

Figure 2. Thymic tissue regeneration results in restoration of the naive and effector T cell compartment later in life. (A) Lymphocyte count of CD3+, CD4+, and CD8+ T cells in HCs and neonatally Tx children older than 10 years of age. (B) Percentages of CD31 expression on CD45RA+CD4+ T cells. (C) Percentages of naive T cells (CD45RA+CCR7+) within the CD4+ T cell population. (D) Percentages of Tem (CD45RA+CCR7+CD28+CD27+FAS+) in CD4+ T cells. Black squares (or black bars), older HCs (n = 10); gray squares (or gray bars), older Tx patients (n = 24–26). Older Tx patients are further divided in high percentages of CD31 (n = 17–19; closed gray square in B) and older Tx with low CD31 percentages (n = 7; open gray squares in B), as described in the x axis. Data are shown as mean ± SD. See also Supplemental Figure 2. *P < 0.05, Mann-Whitney U test used.

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Thus, although functional thymic tissue regeneration and restoration of the naive CD4+ T cell compartment after neonatal thymectomy occur in the majority of children in later life, a subgroup of Tx children show no signs of restoration of thymic output. Naive T cell function is impaired after neonatal thymectomy, but is restored with thymic tissue regeneration in later life. As the naive CD4+ T cell compartment is greatly altered after neonatal thymectomy, we next investigated functional characteristics of the remaining CD4+ naive T cells. Following TCR triggering, naive CD4+ T cells normally show strong calcium fluxes, higher than those found in memory T cells (ref. 26 and Supplemental Figure 3A). When we compared calcium fluxes following CD3 engagement in naive CD4+ T cells there was a striking, significant decrease in the calcium flux in the cells derived from young Tx patients compared to young HC patients (Figure 2C), a lower total naive CD4+ T cell count (P < 0.001, 27 and 192 × 10^6/l, median, Supplemental Figure 2B) and an increased percentage of Tcm (Figure 2D) compared with the Tx individuals with higher percentages of CD31-expressing naive CD4+ T cells. When only focusing on the subgroup of neonatally Tx children with higher CD31 percentages in their naive T cell pool and age-matched young adolescents, no significant differences were noted for absolute naive T cells and Tscm, Tcm, and Tem CD4 numbers (Supplemental Figure 2B) as well as the proportion of naive T cells (Figure 2C), Tcm (Figure 2D), Tem (Figure 2E), and Tscm (Figure 2G) compared with age-matched HCs. Similar data were obtained for CD8+ T cells, except for a proportional increase in CD8+ Tem and no difference in CD8+ Temra, as seen for CD4+ T cells (Supplemental Figure 2, C and D).

Thus, although functional thymic tissue regeneration and restoration of the naive CD4+ T cell compartment after neonatal thymectomy occur in the majority of children in later life, a subgroup of Tx children show no signs of restoration of thymic output.

**Naive T cell function is impaired after neonatal thymectomy, but is restored with thymic tissue regeneration in later life.** As the naive CD4+ T cell compartment is greatly altered after neonatal thymectomy, we next investigated functional characteristics of the remaining CD4+ naive T cells. Following TCR triggering, naive CD4+ T cells normally show strong calcium fluxes, higher than those found in memory T cells (ref. 26 and Supplemental Figure 3A). When we compared calcium fluxes following CD3 engagement in naive CD4+ T cells there was a striking, significant decrease in the calcium flux in the cells derived from young Tx patients compared
We next studied whether functionality of the naive CD4+ T cell was regained with thymic tissue regeneration later in life. In the older Tx group (Tx > 10 yr), with higher CD31 percentages, we indeed observed restored calcium fluxes (Figure 3E) and IL-8 production of naive T cells in the majority of children compared with age-matched HCs (Figure 3F). While IL-8 production decreased with age, the capability of naive CD4+ T cells to produce IL-8 actually was higher in the older Tx group than in the young Tx group (P < 0.0001; 6.39% vs. 1.46% median, respectively) supporting functional rejuvenation of the naive T cell compartment. In patients with a persistence of low percentages of CD31-expressing CD4+ naive T cells, a lower calcium flux persisted (Figure 3E) as did decreased IL-8 production (Figure 3F). These data indicate that the thymus is not only necessary for maintaining naive T cell numbers, but also for functional restoration of the naive T cell compartment.

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Neonatal thymectomy induces the loss of IL-8 expression within the CD31+ naive CD4+ T cell compartment. The naive T cell compartment can be divided based on CD31 expression. The CD31+ fraction is thought to be enriched for RTEs and to represent the “true naive” cells with little or no peripheral proliferative history (16, 17, 27), but little is known about the functional differentiation between the CD31+ and CD31− naive CD4+ T cell subset. In young Tx children, a significant decrease in the percentage of CD31+ and an increase in the percentage of CD31− naive CD4+ T cells in the CD4+ T cell compartment was observed compared with that in HCs (Figure 4A). In young HCs, expression of IL-8 by naive CD4+ T cells was largely confined to the CD31+ naive CD4+ T cell fraction (Figure 4B) and strongly reduced in both the CD31+ and CD31− naive T cell compartments of young Tx children (Figure 4B). The loss of IL-8 in young Tx children was accompanied by an increase in IFN-γ expression (Supplemental Figure 4). The CD31− naive CD4+ T cell fraction had an overall higher IFN-γ expression compared with the CD31+ fraction of both the Tx and HC group, but CD31+ naive T cells of young Tx children had a significantly higher expression of IFN-γ compared with age-matched HCs (Supplemental Figure 4). In older Tx patients, restoration of functional thymic tissue resulted in the restoration of IL-8 production by CD31+ naive CD4+ T cells compared with HCs and was even higher than in young Tx patients, while in older Tx patients with low CD31+ naive T cell numbers, no such restoration was seen (Figure 4C). Together, these data indicate that IL-8 production is predominantly enriched in the CD31+ naive CD4+ T cell compartment, and in vitro analysis suggests that this functional heterogeneity within the CD31+ naive CD4+ T cell compartment is cell division related.

CD31+ naive CD4+ T cells of Tx children are distinct from those of HCs and resemble their CD31− naive CD4+ T cell counterparts. To further assess any transcriptional differences between naive T cells of HCs and Tx children, we performed gene-expression profiling by RNA sequencing. We analyzed the transcriptome of both FACS-sorted CD31+ and CD31− naive CD4+ T cells from Tx children (n = 4, between 1 and 5 years after Tx) and age-matched HCs (n = 3) (Supplemental Figure 6). Unsupervised cluster analysis showed that both CD31+ and CD31− naive CD4+ T cell subsets of HCs did not cluster with their counterparts in Tx children (Fig-
ure 5A). While within the group of young HCs, no specific clustering was noticed regarding both naïve T cell subsets, in young Tx children, the CD31+ and CD31- naïve subsets clustered together within the same patient (Figure 5, A and B). Thus CD31- naïve T cells of Tx children now resembled their CD31+ naïve counterparts, indicating loss of heterogeneity. However, both naïve subsets of Tx children were still distinct from the CD31+ and CD31- naïve T cell subsets of HCs and especially memory T cells of either the HC or Tx group (Figure 5B and data not shown respectively). This suggests that both naïve T cell subsets may undergo transcriptional changes after thymic removal, leading to converging transcriptional profiles.

Transcript analyses resulted in 305 targets that significantly differed between CD31+ naïve CD4+ T cells of Tx children and HCs (Supplemental Table 1). In contrast, only 2 other transcripts (MAF and FAM129C), next to CD31 (PECAM-1) were found to differ between CD31+ and CD31- naïve CD4+ T cells of Tx children (Supplemental Table 2), while 25 transcriptional differences were present in HCs (Supplemental Table 3). This further demonstrates that, after neonatal thymectomy, CD31- naïve CD4+ T cells resemble CD31+ naïve CD4+ T cells despite expression of CD31. Pathway enrichment analysis showed several pathways that were all significantly different (Supplemental Figure 7A) and all upregulated in the CD31+ naïve T cells of Tx children, including nucleotide metabolism, cytokine signaling, apoptosis, and NF-κB signaling, indicating a more activated and/or proliferated state of CD31+ naïve CD4+ T cells in Tx patients compared with HCs (Supplemental Figure 7B). Together, these data indicate that there is transcriptional differentiation within the naïve CD4+ T cell compartment, which is lost following neonatal thymectomy.

Discussion

Naïve CD4+ T cells are the source of the effector/memory T cell compartment and are considered relatively homogeneous as opposed to the effector/memory T cell population. To stay truly naïve, naïve T cells have to avoid differentiation and remain relatively quiescent. With human aging, thymic output and naïve CD4+ T cell numbers decline, but it has not been clear whether this is accompanied by (functional) differentiation of naïve T cells and loss of true naïvety. By utilizing a unique cohort of neonatally Tx children, we here demonstrate functional differentiation within the human naïve CD4+ T cell compartment, which is lost following neonatal thymectomy. In addition, functional thymic regeneration was seen in the majority of neonatally Tx patients and resulted in restoration of functional differentiation and rejuvenation of the naïve CD4+ T cell compartment, overall providing new insights in naïve T cell dynamics that result in the better identification of “truly naïve” cells and RTEs.

Heterogeneity within the naïve CD4+ T cell compartment was already established by the expression of CD31 (16, 17). We now demonstrate further functional differentiation within CD31- naïve CD4+ T cells on the basis of IL-8 production and its thymic dependence. IL-8 production was preferentially seen in the PTK7 fraction, but declined after loss of PTK7 due to cell division in vitro. While in mice, maintenance of the peripheral naïve T cell compartment is primarily dependent on thymic output, human adults sustain naïve T cell numbers by peripheral proliferation (9).

Several studies have suggested that even in young, HCs, a substantial proportion of naïve T cells are derived from peripheral T cell renewal (4, 29). Thymic contribution might, however, be more prominent than initially thought, as thymic regeneration restored naïve T cell numbers, naïve T cell function, and phenotype, while even a decade after neonatal thymectomy, these remained altered and distinct from those of HCs in the absence of thymic regeneration. In either case, and most importantly, the thymus seems responsible for the functional differentiation of the naïve T cells. Therefore, this study highlights that pediatric naïve T cell function is not similar to that of adults and puts further constraints on the extrapolation of insights into naïve T cell function from children to adults and vice versa.

Throughout life, the peripheral T cell pool is maintained at a relatively constant size. As thymic export stops or diminishes, the maintaining peripheral T cell pool proliferates, replenishing the peripheral space and gradually acquiring an effector/memory phenotype, as also seen in lymphopenic conditions and aging (8, 9, 13, 30). This slowly results in the loss of naïve T cells and skewing toward the effector/memory phenotype. After neonatal thymectomy, we detected an increase in proliferation of T cells as well as skewing toward a Tcm and Tem phenotype, likely at the expense of naïve T cells. Of interest, an increased proportion of a newly reported T cell subset (25) called Tscm is seen after neonatal thymectomy. These Tscm are suggested to be long lived and endowed with differentiation potential and self-renewal abilities, as they can generate effector/memory T cell subsets while preserving the original naïve-like phenotype (25). Recent human in vivo analysis has suggested that relative Tscm frequencies do not vary much with age, but could increase to compensate for reduced naïve T cell output (31). The increased Tscm compartment after neonatal thymectomy could therefore be compensatory for the reduced naïve T cell compartment and aid in the expansion of the Tcm and Tem compartments to normal levels. Its actual contribution is, however, uncertain, as Tscm numbers are decreased in Tx children despite their increased proportion in CD4+ T cells.

During normal T cell activation, T cell antigen receptor (TCR) ligation results in rapid cytoskeletal rearrangements and a dramatic increase of intracellular calcium (Ca2+) concentration (32). Thus, Ca2+ signaling is essential for T cell activation and T cell–dependent immune responses. After neonatal thymectomy, a drastic decrease in calcium flux was seen. Not only the height of the flux was decreased; the calcium flux was also delayed in comparison with that of HCs. The potential for an adequate calcium flux was, however, not affected, as stimulation with ionomycin resulted in an equal maximum calcium peak. In addition, T cell activation–dependent production of IL-2 was not diminished, demonstrating that, despite an altered calcium flux response, naïve T cells were still responsive. A delayed calcium flux response in naïve CD4+ T cells is not only apparent after neonatal thymectomy, but also with increasing age (26). This delay we observed was not due to an increased proportion of CD31+ naïve T cells; even though these cells do show lower calcium flux compared with the total CD4 naïve T cell population, their calcium response was not delayed (data not shown). Together, these data indicate features reminiscent of maturation and functional differentiation within the naïve T cell compartment after neonatal thymectomy.
Another indication of an altered naive T cell compartment was the loss of IL-8 production that we observed in naive T cells of Tx children. IL-8 production has recently been revealed as a signature effector function of naive/immature CD4+ T cells in newborns. We here show that IL-8–producing naive T cells are also present in infants, but decline with age, as documented for adults (2). The almost complete loss of IL-8 and concomitant increase in IFN-γ production of naive CD4+ T cells after neonatal thymectomy indicates a functionally altered naive T cell compartment, resembling that of the adult immune system. While the production of IL-8 of CD31+ naive CD4+ T cells was strongly dependent on thymic presence, SP CD4+ thymocytes barely produced any IL-8. When we further subdivided the CD31+ naive population based on PTK7 expression, we found an enrichment of IL-8–positive cells within the PTK7–CD31+ naive RTE CD4+ T cells. This suggests that there is a very small window of differentiation in which IL-8 expression is induced. The loss of IL-8 expression could be induced by a mixture of cytokines that also downregulates PTK7 (28) while maintaining CD31 expression, indicating that non-antigen–driven proliferation is sufficient to induce functional differentiation of RTEs.

CD31 has been shown to identify a compartment enriched in RTEs, whereas CD31– naive T cells are thought to arise following homeostatic proliferation (16, 17). When comparing gene-expression profiles of CD31+ naive CD4+ T cells of both HCs and Tx children, large differences were found (>200 genes differentially expressed) associated with pathways in T cell activation. More importantly, transcriptional analysis of CD31+ and CD31– naive T cells from Tx children revealed an almost complete overlap between the 2 populations, which was not present in HCs. This demonstrates that after neonatal thymectomy, CD31+ T cells undergo differentiation toward CD31+ cells without the loss of CD31, supporting the observed reduction in IL-8 production and calcium flux of naive T cells after thymectomy. The phenomenon of partial differentiation of naive T cells in aging has also been suggested based on differential expression of miRNAs (miR-181a, miR146a, and miR-21) (27) and CD25 expression on naive CD4+ T cells (33). Hence, with neonatal thymectomy and with aging, naive T cells may acquire features of memory cells with maintenance of their naive phenotype. This partial differentiation may have detrimental consequences because the naive T cells seem to have lost what is essential for their function, while not having fully gained their new differentiation state. In addition, this also greatly affects our current understanding for identifying “truly naive” T cells and RTEs, as CD31+ cells can be maintained on naive T cells even though they functionally resemble CD31– naive T cells.

While thymectomy could simply result in the gradual loss of RTE phenotypes (high IL-8 producers), multiple other factors, such as (lack of) thymic hormones, (selective) homeostatic proliferation, and an altered cytokine environment, could play a role in the altered phenotypical and functional characteristics of the naive T cell compartment following neonatal thymectomy. For instance, IL-7 plays an important role in T cell number maintenance and is used as therapy to enhance T cell reconstitution (34). The effects of IL-7 are most potent on RTEs, in which IL-7–mediated signaling can induce proliferation in the absence of TCR signaling (35, 36). We have previously shown that IL-7 is increased following thymectomy (23). We now show that cytokine stimulation can induce the loss of PTK7 and IL-8 expression in naive T cells in vitro. Hereby, IL-7 therapy could increase naive T cell numbers, but with the potential loss of IL-8–producing naive T cells within the naive T cell compartment. In addition, IL-7–mediated induction of T cell proliferation in response to low-affinity self antigens is essential for the homeostatic proliferative response observed during lymphopenia (37, 38). Increased (selective) homeostatic proliferation following lymphopenia has indeed been associated with increased development of autoreactivity and subsequent autoimmunity. Even though increased T cell proliferation is evident after thymectomy, it is still unknown whether neonatal thymectomy results in homeostatic proliferation based on self-antigen recognition and whether this differs between CD31+ and CD31– naive T cells.

We have previously reported that, for a majority of the children, evidence of thymic tissue regeneration is observed 5 to 10 years after thymectomy (23). We assessed thymic tissue regeneration and thymic output by MRI imaging and percentage of CD31 in naive CD4+ T cell measurements. In a previous cohort, a similar proportion of children showed regeneration of thymic tissue assessed by MRI scan (23). However, the presence of thymic tissue does not have to indicate a functional thymus, and lack of thymic tissue cannot always be adequately determined. We therefore assessed functional thymic tissue regeneration primarily via CD31 measurement. In our cohort, all patients with lower CD31+ naive T cells did not show any thymic tissue on MRI, while only a small portion of children with higher CD31 did not present evident thymic tissue on MRI. While regeneration of thymic tissue was likely due to regrowth of residual thymic tissue, as it was found to be in the same location prior to thymic removal, the presence of additional cervical thymi, as has been observed in mice (39, 40), could not be assessed. Thymic tissue regeneration, assessed by increased percentages of CD31+ naive T cells, was associated with phenotypical restoration of the naive T cell compartment as well as functional restoration of the calcium flux and neonatal effector cytokine production of IL-8. It has been recently shown in mice that neonatal Tregs are functionally and phenotypically distinct from those produced later in life (41), indicating that there may be unknown qualitative changes in the restored naive CD4+ T cell compartment (e.g., an altered TCR repertoire). In a quarter of the children, no evidence for functional thymic tissue regeneration was detected after at least a decade. These children continued to have a disturbed phenotypical and functional naive/effector compartment, comparable to children in their first years after thymectomy. The rejuvenation capacity of the thymus is an interesting option to explore, and many interventions have been tried to restore thymic function in the elderly or in a setting of hematopoietic stem cell transplantation. Although to date, no interventions fully restore thymic function in the aging host, systemic administration of various cytokines and hormones or bone marrow transplantation have resulted in a degree of increased thymic activity and T cell output with age (42–45). Why thymic tissue regenerates in some children and not in others is unclear and warrants further investigation. It has been hypothesized that the age at which the thymectomy is performed might play a role in the regenerative capability later in life, as some studies with children thymectomized at an average age of 2.6 years still reported diminished naive T cell counts and naive T cell TREC contents well into the...
third decade of life (19, 46). In the current cohort, all children were thymectomized within the first month of life, and this resulted in a proportion (73%) of thymic regeneration similar to that seen in our previous cohort (81%), in which most children were thymectomized within the first 4 months of life (23). Besides the age of thymectomy or possible differences in soluble factors stimulating thymic regeneration, the extent of minor thymic tissue remnants may be an important factor for tissue regeneration.

In conclusion, this study shows that the thymus has a key role in the phenotypical and functional maintenance of the naive CD4+ T cell population and is able to functionally regenerate later in life. We propose that thymic tissue should be preserved as much as possible during cardiac surgery, as not all children regenerated thymic tissue. Additionally, these findings give further insight in the functional heterogeneity of the naive T cell compartment by identifying differential IL-8 production within the CD31+ naive CD4+ T cell compartment and the dependence of this differentiation on thymopoiesis. This appreciation could aid us in better monitoring thymopoiesis and further understanding the mechanisms of naive T cell homeostasis after, for instance, immune ablative therapy as well as in aging.

Methods

Study design and population. Patients who had undergone complete thymectomy within the first month of life because of surgery to treat congenital heart defects at the Wilhelmina Children’s Hospital were included in this study. Surgeries involving defects in the major vessels, such as transposition of the great arteries, hypoplastic left heart syndrome, and hypoplastic arch with or without coarctation of the aorta, routinely necessitate thymectomy. An overview of the study population is described in Table 1. Blood samples were taken at between 1 to 5 years (patients referred to as young Tx or Tx 1–5 yr) and after 10 years (patients referred to as older Tx or Tx > 10 yr) following neonatal thymectomy, since we have previously shown that the potential to regenerate thymic tissue occurs at approximately 5 to 10 years. (23). Patients showed no clinical signs of infection at the time of inclusion and of memory CD4+ T cells on CD3+CD8–CD45RA– T cells (CD45RA μ′)2 fragments on other fluorochromes other than CD3 could potentially interfere with the analyses against human CD8 (Sk-1) and FAS (CD95, clone DX2), and Ki-67 (B56) were from BD Biosciences, and CD3 (UCHT1), CD4 (RPA-T4), and CD45RA (HI100) were from BioLegend. Finally, stained mononuclear cells were washed twice in FACS buffer, run on a FACSCanto II, and analyzed by using FlowJo software (TreeStar).

As cell counts and/or sufficient cells were not available for all samples due to the limited amount of blood allowed to be taken from children, some data points are not shown for all study subjects.

Cells of infectious diseases or a hematologic or immunologic disorder.

Calcium flux. T cells were purified from fresh PBMCs by magnetic bead separation using the biotin human T lymphocyte enrichment cock- tail (BD IMag, BD Biosciences) according to the manufacturer’s instructions. Purity of MACS-sorted CD3+ T cells was greater than 90%.

500,000 MACS-isolated T cells were incubated with 0.2 mg/ml Fluo-3 and 0.4 mg/ml Fura Red (Invitrogen) for 30 minutes at 37°C. During the last 10 minutes, a combination of the FACS antibodies CD3 (OKT3, BioLegend), CD8 (SK1, BD Bioscience), and CD45RO (UCHL1, Biolegend) was added. Cells were washed twice and resuspended in HBSS supplemented with FCS. Baseline cytosolic calcium levels were measured on a FACSCanto II flow cytometer. Next, 20 μg/ml anti-IgG F(ab′)1, fragments (Jackson Immunoresearch) were added, and calcium flux was measured for 4.5 minutes. Subsequently, 0.1 mg/ml ionomycin (Calbiochem) was added. Calcium flux of CD4+ naive T cells was assessed by gating on CD3+CD8 CD45RO ‘T cells and of memory CD4+ T cells on CD3+CD8 CD45RA ‘T cells (CD45RA [HI100, BioLegend]), as binding of anti-IgG F(ab′)2 fragments on other fluorochromes other than CD3 could potentially interfere with the CD3-triggered calcium flux.

T cell stimulation. 200,000 PBMCs were stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) for 5 hours at 37°C and 5% CO2 in culture medium (10% hAB-serum 1% penicillin, streptomycin, and 1% l-glutamine). After 1 hour of culture, GolgiStop (BD) was added to the culture. Cells were then washed and blocked with normal mouse serum followed by extracellular staining, fixation in Cytofix/Cytoperm, and

### Table 1. Patient characteristics

<table>
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<td>Number of patients</td>
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<td>17</td>
<td>11</td>
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<tr>
<td>Age at thymectomy (d)</td>
<td>–</td>
<td>8 (7.93)</td>
<td>–</td>
<td>7 (3.92)</td>
</tr>
<tr>
<td>Age at blood draw (yr)</td>
<td>2.04 (1.00)</td>
<td>2.12 (1.16)</td>
<td>13.70 (1.48)</td>
<td>15.89 (4.16)</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>13 (68.4%)</td>
<td>11 (64.7%)</td>
<td>7 (63.6%)</td>
<td>17 (65.4%)</td>
</tr>
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Data are shown as median ± SD for age at of thymectomy in days and age at blood sampling in years. Numbers (percentages) are shown for male sex.
washing in Perm/Wash solution (Cytotox/Cytoperm Kit, BD). Finally, cells were blocked with normal rat serum and incubated with the appropriate mAbs against IL-2 (MQ1-17H12, BD Biosciences) and IL-8 (BH0814, Biolegend) for intracellular cytokine staining.

In the case of measurement of IL-8 production in thymus (SP CD4+ thymocytes, CD3+CD4+CD8-) and PBMCs (CD31+CD45RA-CD8-CD4+CD3+ T cells), samples were gathered from the same neonate. Thymocytes were isolated via staining through a mesh filter; this was followed by Ficoll Isopaque density gradient centrifugation. PMA/ ionomycin stimulation was performed as described above and on fresh isolated cells.

In vitro cytokine stimulation. CD31+ naive T cells were FACS sorted (Supplemental Figure 6) and stained with CellTrace Violet (final concentration 2 μM) for 7 minutes at 37°C. The reaction was stopped by adding 10× volume of cold, filtered 100% FCS. Cells were washed and placed at 50,000 T cells per well in culture medium, followed by stimulation with 25 ng/ml of recombinant human IL-6 (BD Biosciences — Pharmingen), IL-7 (ImmunoTools), IL-10 (ImmunoTools), IL-15 (ImmunoTools), and IFN-γ (eBioscience) as previously described, for 6 days (28). After cytokine stimulation, cells were washed and stained with PMA and ionomycine as described under T cell stimulation to determine IL-8 and IFN-γ cytokine production. PTK7 (Miltenyi Biotec, 188B) and isotype staining (Miltenyi Biotec, S43.10) were performed to assess PTK7+ cells in addition to CD3, CD4, CD31, and CD45RA cells.

RNA sequencing. PBMCs from neonatally Tx children (1–5 years of age, n = 4) and age-matched controls (1–5 years, n = 3) were FACS sorted on CD31+ and CD31 naive CD4+ T cells using the following mAbs: CD3 (clone OKT3, BioLegend), CD4 (clone RPA-T4, eBioscience), CD25 (clone BC96, BioLegend), CD45RA (clone HI100, BioLegend), CD45RO (cloneUCHL1, BioLegend), CD31 (clone WMS9, BioLegend), CD127 (clone eBioRDR5, eBioscience), and CCR7 (clone 150503, R&D Systems). To ensure that no contamination of Tregs and immuno-myocytes as described under T cell stimulation to determine IL-8 and IFN-γ cytokine production. PTK7 (Miltenyi Biotec, 188B) and isotype staining (Miltenyi Biotec, S43.10) were performed to assess PTK7+ cells in addition to CD3, CD4, CD31, and CD45RA cells.

Total RNA was purified from sorted CD31+ and CD31 naive CD4+ T cells using the AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN) according to the manufacturer’s instructions. RNA was stored at −80°C until further processing. mRNA was isolated using Poly(A) Beads (NEXTflex). Sequencing libraries were prepared using the Rapid Directional RNA-Seq Kit (NEXTflex) and sequenced on Illumina NextSeq500 to produce single-end 75 base long reads (Utrecht DNA Sequencing Facility). Sequencing reads were mapped against the reference genome (hg19 assembly, NCBI37) using BWA (47) package (mem –t 7 –c 100 –M). Reads mapping to multiple locations were excluded from the analysis. The RPKM values and the raw read numbers were calculated using the rnaSeq_countgeneread function from Cisgenome v2 (48).

Statistics. Statistical significance between 2 groups was assessed using the Mann-Whitney U test for unpaired data and Wilcoxon’s signed rank test for paired data. In the case of the cytokine stimulation of CD31+ naive CD4+ T cells, Kruskall-Wallis test was performed (2-sided), and comparisons were considered significant at P < 0.05 after Dunn’s multiple comparison test. Differentially expressed genes were identified using the DESeq2 package with standard settings (49). Genes with log2 fold change larger than 0.6, base mean greater than 10, and P value adjusted at less than 0.05 (False Discovery Rate B & H) were considered as differentially expressed. In the correlation heat map, Pearson’s correlation was used as an index of similarity. Pathway enrichment analysis was clustered by ToppFun (https://toppgene.cchmc.org/enrichment.jsp)

Accession numbers. All original microarray data were deposited in the NCBI’s Gene Expression Omnibus (GEO GSE72400).

Study approval. The study was approved by the medical ethical committee of the University Medical Center Utrecht, and written consent was obtained from all study participants or their legal guardians prior to inclusion in the study in agreement with the Helsinki Declaration of 1975 (revised in 1983).

Author contributions. TVDB and FWV were responsible for conceptualization. TVDB, EMD, and FWV were responsible for methodology. Investigation was performed by TVDB and EMD. Formal analysis was performed by TVDB and MM. TVDB and FWV wrote the original draft. Resources were provided by WMJ, MM, RAJN, EESN, and JCB. Supervision was by FWV, NJGJ, KT, JAMB, and BPJ. BPJ and FWV were responsible for funding acquisition.

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