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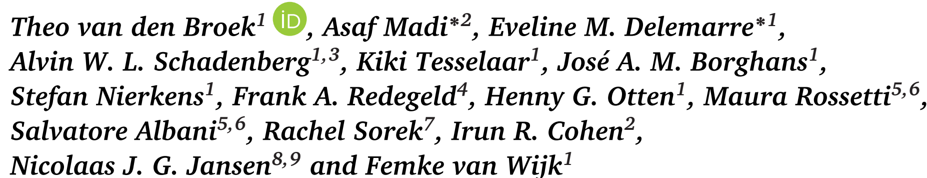
Research Article

Human neonatal thymectomy induces altered B-cell responses and autoreactivity


Theo van den Broek[1], Asaf Madi[2], Eveline M. Delemarre*[1], Alvin W. L. Schadenberg[1,3], Kiki Tesselaar[1], José A. M. Borghans[1], Stefan Nierkens[1], Frank A. Redegeld[4], Henny G. Otten[1], Maura Rossetti[5,6], Salvatore Albani[5,6], Rachel Sorek[7], Irun R. Cohen[2], Nicolaas J. G. Jansen[8,9] and Femke van Wijk[1]*

[1] Laboratory of Translational Immunology, University Medical Center Utrecht/Wilhelmina Children's Hospital, Utrecht, The Netherlands
[2] Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel
[3] Department of Pediatric Intensive Care, Bristol Royal Hospital for Children, Bristol, UK
[4] Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
[5] Duke-National University of Singapore Graduate Medical School, Singapore
[6] SingHealth Translational Immunology and Inflammation Centre, SingHealth, Singapore
[7] ImmunArray Ltd., Rehovot, Israel
[8] Department of Pediatric Intensive Care, University Medical Centre Utrecht, Utrecht, The Netherlands
[9] Department of Pediatric Cardiothoracic Surgery, University Medical Centre Utrecht, Utrecht, The Netherlands

An association between T-cell lymphopenia and autoimmunity has long been proposed, but it remains to be elucidated whether T-cell lymphopenia affects B-cell responses to autoantigens. Human neonatal thymectomy (Tx) results in a decrease in T-cell numbers and we used this model to study the development of autoreactivity. Two cohorts of neonatally thymectomized individuals were examined, a cohort of young (1–5 years post-Tx, n = 10–27) and older children (>10 years, n = 26), and compared to healthy age-matched controls. T-cell and B-cell subsets were assessed and autoantibody profiling performed. Early post-Tx, a decrease in T-cell numbers (2.75×10^9/L vs. 0.71×10^9/L) and an increased proportion of memory T cells (19.72 vs. 57.43%) were observed. The presence of autoantibodies was correlated with an increased proportion of memory T cells in thymectomized children. No differences were seen in percentages of different B-cell subsets between the groups. The autoantigen microarray showed a skewed autoantibody response after Tx. In the cohort of older individuals, autoantibodies were present in 62% of the thymectomized children, while they were found in only 33% of the healthy controls. Overall, our data suggest that neonatal Tx skews the autoantibody profile. Preferential expansion and preservation of Treg (regulatory T) cell stability and function, may contribute to preventing autoimmune disease development after Tx.

Keywords: Autoimmunity · B cells · Homeostatic proliferation · Lymphopenia · Regulatory T cell · Thymectomy

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Correspondence: Femke van Wijk
e-mail: F.vanwijk@umcutrecht.nl

*These authors contributed equally to this work.

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Introduction

The thymus is the major production site of T cells, starting at around 12 weeks of gestation [1]. After the first years of life its function diminishes, but is maintained into adulthood although at a much lower level [2]. In adulthood, T-cell homeostasis is mainly provided by peripheral homeostatic proliferation (HP) [3]. However, the role of the thymus in T-cell homeostasis is probably much more prominent very early in life; indeed, neonatal thymectomy (Tx) has been shown to result in T-cell lymphopenia and in skewing toward a memory phenotype [4–7]. The thymus is necessary for the development of self-tolerance but it is unknown whether neonatal Tx leads to enhanced autoreactivity. In neonatal cardiac surgery, the thymus, which obstructs access to the heart and great vessels, is routinely removed. Except for transient lymphopenia, these children show no clinical signs of immune deficiency or immune deregulation [6, 8]. Subsequent to neonatal Tx, antibody titers to previous vaccinations and responses toward T-cell mitogens are relatively unaltered [9, 10]. However, responses to new vaccinations (e.g., against tick-borne encephalitis and hepatitis B) seem to be delayed and sometimes absent [11, 12]. This suggests that B-cell homeostasis and the antibody repertoire may be affected by neonatal Tx, likely due to altered T-cell help.

Thymopoiesis results in the generation of new naïve T-cell specificities, while HP expands the existing peripheral T-cell pool. HP is driven by cytokines and in part by recognition of self-peptide/MHC ligands [13, 14]. Recovery from T-cell lymphopenia, via HP, may therefore result in expansion of autoreactive T cells and could result in some degree of loss of self-tolerance and alterations in the autoantibody repertoire. An association between T-cell lymphopenia and autoimmune disease is recognized, as several autoimmune diseases are associated with low T-cell numbers [15]. Furthermore, in animal models lymphopenia is a factor that drives the development of autoimmunity [16, 17]. However, the occurrence of autoimmunity has been considered as at least a two-hit model that requires a cofactor next to homeostatic T-cell proliferation, such as a disruption in inhibitory factors [18]. For instance, regulatory T (Treg) cells play an important role in the control of self-reactivity and have been shown to affect lymphopenia-induced proliferation [18]. It is currently unknown whether Tx at early age in an otherwise healthy person will result in increased autoreactivity and whether such autoreactivity necessarily leads to autoantibodies and autoimmune disease.

The thymus has a clear role in the development of the T-cell repertoire, but also indirectly affects the B-cell repertoire via T-cell help and suppression. Follicular T helper cells (Tfh) are effector T cells that are specialized in providing B-cell help. Tfh cells home to B-cell areas in secondary lymphoid tissue using their chemokine receptor CXCR5 and its ligand CXCL13. Tfh cells and their production of IL-21 are essential for germinal center formation, affinity maturation, and the development of most high-affinity antibodies and memory B cells [19]. However, several autoimmune diseases and higher titers of circulating autoantibodies are associated with increased frequencies of circulating Tfh cells and increased concentrations of CXCL13 [20]. Different B lymphocyte subpopulations can be identified and various antibody disorders have been related to alterations in their distribution. For instance, the loss of CD21 or increased CD5 expression on B cells has been reported to be associated with autoimmune disease [21–23]. While IgG autoreactive antibodies are often associated with autoimmune disease, IgM autoreactive antibodies have been associated with maintenance of self-tolerance [24, 25]. Cohen and colleagues previously showed that the IgM autoantibody repertoire is shared by most newborns and is primarily directed to relatively uniform sets of self-antigens. The IgM autoantibody reactivities of their healthy mothers showed a significantly lower overlap with their newborns [26, 27]. This suggests that the natural autoantibody repertoire of humans begins with a standard set of autoreactive antibodies, which later diverge as a result of individual immune experience during life. The role of the thymus in the developing antibody repertoire remains to be elucidated.

Here, we studied a unique cohort of neonatally thymectomized children to assess the role of the thymus in T-cell and B-cell subset homeostasis as well as the change in the autoantibody repertoire upon Tx. In addition, we assessed the role of thymic tissue regeneration, which has been shown to occur in the majority of neonatally thymectomized children later in life [4, 6]. Using this cohort, we now show that in the first years after neonatal Tx, the absolute number of T cells is decreased, while the proportion of memory T cells, circulating Tfh cells and plasma concentration of CXCL13 are increased. The (auto) antigen microarray showed a skewed autoantibody response after Tx, but none of the individuals manifested with overt autoimmune disease. Preferential expansion of Treg cells after neonatal Tx was found, which may play a role in suppressing autoreactivity from becoming clinical overt. Lack of thymic tissue regeneration later in life did not correlate with the presence of selected autoantibodies. Together, these findings imply that neonatal Tx results in skewing of the autoantibody repertoire early in life, but does not seem to result in clinical autoimmune disease in our cohort, which might be due to expansion of Treg cells and relative maintenance of naïve Treg cells.

Results

Neonatal thymectomy directly affects T-cell numbers and proportions

We first examined T-cell lymphopenia in a cohort of children 1–5 years following neonatal Tx (young Tx). In this Tx group, T-cell numbers were significantly lower than in healthy age-matched controls (young HC) (Fig. 1A). Within the T-cell compartment, both the proportion and the absolute number of naïve T cells were significantly reduced (Fig. 1B, left and right panel, respectively). Concurrently, there was an increase in the proportion of memory T cells in young Tx children (Fig. 1C, left panel), although their absolute numbers of memory T cells were lower than in age-matched HC (Fig. 1C, right panel). The expression of ki-67, a marker associated with cell proliferation, by $CD3^+$ T cells, was significantly increased in these thymectomized children (Fig. 1D), while the

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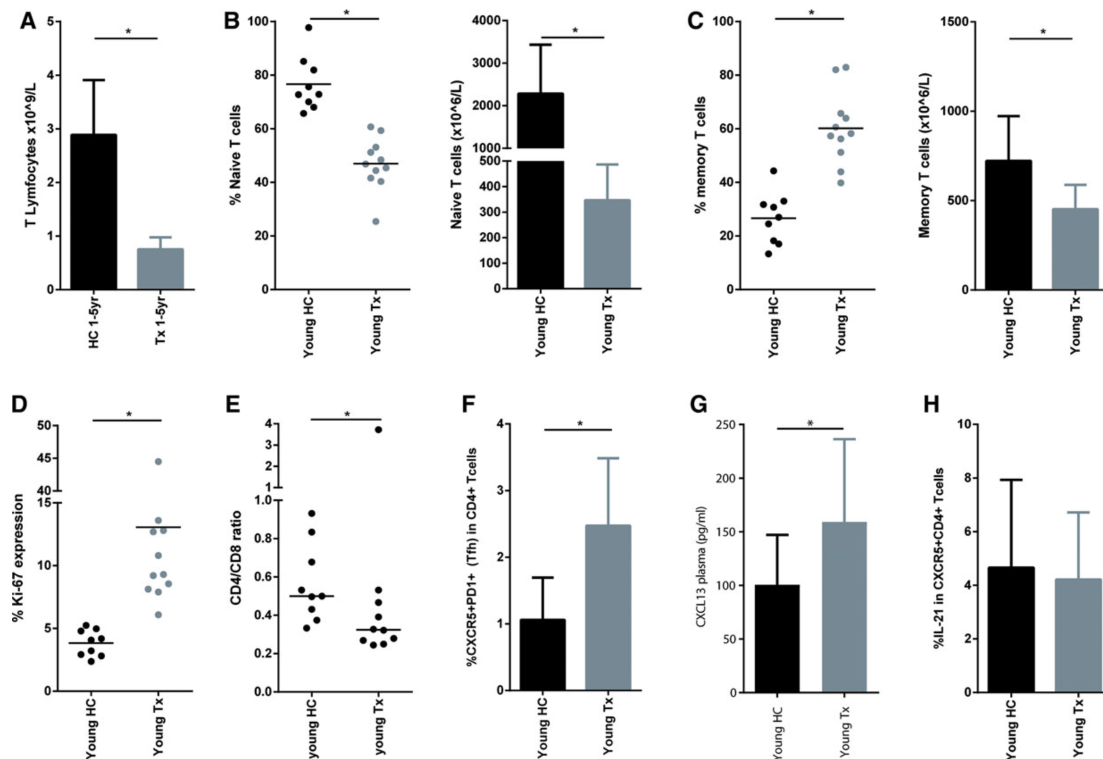

Figure 1. Neonatal Tx results in lymphopenia-induced T-cell proliferation.
PBMCs and plasma were isolated from heparinized blood samples and analyzed by flow cytometry and Luminex, respectively. (A) T Lymphocyte count in healthy controls (young HC, $n = 9$) and thymectomized (young Tx, $n = 10$) children. (B) Proportion and numbers of naïve ($CD45RA^+CCR7^+$) $CD3^+$ T cells in HC ($n = 9$) and Tx ($n = 11$) (left and right panel). (C) Proportion and numbers of memory ($CD45RO^+$) $CD3^+$ T cells in HC ($n = 9$) and Tx ($n = 11$) (left and right panel) (D) Percentage of ki-67^+ in $CD3^+$ T cells of HC ($n = 9$) and Tx ($n = 11$) (E) CD4:CD8 ($CD3^+$) ratio in HC ($n = 9$) and Tx ($n = 10$) (F) Tfh ($CXCR5^+PD\text{-}1^+$) in $CD4^+$ T cells in HC ($n = 9$) and Tx ($n = 11$). (G) Plasma CXCL13 levels (pg/mL) of healthy control (young HC, $n = 19$) and thymectomized (young Tx, $n = 11$) children. (H). IL-21 expression in Tfh-like cells in HC ($n = 9$) and Tx ($n = 11$). Panels A–F and H: Data are pooled from three independent experiments with three to five samples per experiment. Mean shown, error bars indicate SD. Panel G, data are pooled from one experiment with 30 samples. Median is shown and error bars indicate SD. Statistical analysis: Mann–Whitney U test. $^*p < 0.05$.

ratio of $CD4^{(+)}$:$CD8^{(+)}$ T cells was significantly reduced (Fig. 1E). Cytokine expression by $CD4^+$ T cells in young Tx children was significantly higher for IFN-γ, IL-17, and IL-13 compared to healthy controls (Supporting Information Fig. 1a) as well as TNFα expression by $CD8^+$ T cells (Supporting Information Fig. 1b).

Upon antigen activation, Tfh are necessary to help B cells generate specific antibodies. The percentage of circulating $CXCR5^+PD1^+$ (Tfh) cells was increased in thymectomized children (Fig. 1F, Supporting Information Fig. 1c) as well as plasma levels of CXCL13, the ligand for CXCR5 (Fig. 1G). No difference in IL-21 expression by Tfh(-like) cells or in IL-21 plasma levels between the young Tx and HC group was detected (Fig. 1H and Supporting Information Fig. 1d, respectively).

Overall, neonatal Tx resulted in a decrease in T-cell numbers, with a concomitant increase in ki-67 expression, skewing toward the memory compartment, and a more proinflammatory cytokine expression profile. In addition, thymectomized children had an increased proportion of circulating Tfh-like cells and an increased concentration of plasma CXCL13.

ANA and ANCA autoantibodies postthymectomy correlate with high percentage of $CD4^+$ memory T cells

To assess the presence of autoantibodies after Tx, a selection of autoantibodies commonly used in clinical diagnostics was measured. In six of 12 thymectomized children, autoantibodies were detected against nuclear antigen (antinuclear antibody (ANA), $n = 4$, patient 8, 9, 11, and 12) and neutrophil cytoplasmic antibodies (antineutrophil cytoplasmic antibody (ANCA), $n = 2$, patient 3 and 7) (Supporting Information Table 1). These six autoantibody positive children had a significantly higher proportion of memory T cells than the autoantibody-negative thymectomized children (p0.003, median 64.8 vs. 36.35%) and than the healthy controls ($p < 0.000$, median 13.1%) (Fig. 2). From two of six autoantibody positive children (patients 3 and 9) a prior sample (respectively, 11 and 37 months earlier) was available. At that earlier time point, both children were still autoantibody negative (see the connecting line in Fig. 2), but their proportions of memory T cells were already increased (Supporting Information

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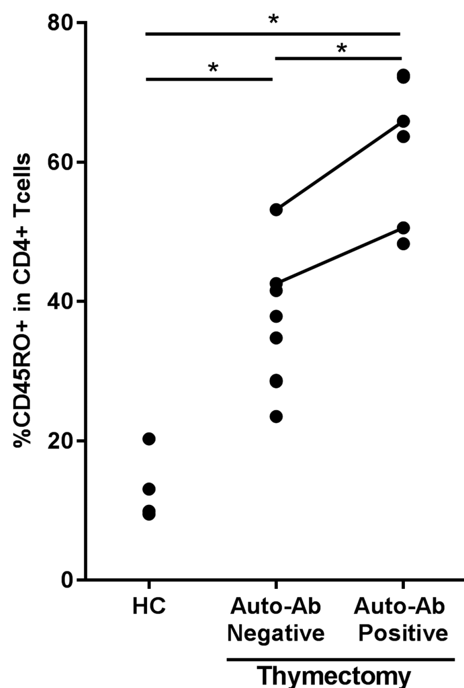

Figure 2. Memory T-cell expansion precedes development of autoreactivity.
PBMCs and plasma were isolated from heparinized blood samples and analyzed by flow cytometry and autoantibody screening, respectively. Percentage memory (CD45RO$^+$) CD4 T cells in HC ($n = 9$), autoantibody negative ($n = 8$) and positive ($n = 6$) young Tx patients. (Line indicates same patient (patients 3 and 9), but at different time). Data are pooled from three independent experiments with three to five samples per experiment. Statistical analysis: Mann–Whitney U test. $^*p < 0.05$.

Table 1). This suggests that memory T-cell expansion occurred prior to the formation of autoantibodies.

Altered autoantibody profile following neonatal thymectomy

To assess the IgM and IgG autoantibody profile after neonatal Tx, we studied plasma using an antigen microarray containing 911 different antigens. A total of 68 autoantibodies were found to be altered in the young Tx group compared to the healthy children. Cluster analysis of the differential autoantibody reactivities between the two groups resulted in two autoantibody reactivity clusters (Fig. 3). Cluster 1 showed a lower autoantibody intensity in thymectomized children; these decreased autoantibodies consisted mostly of IgM autoantibodies. In contrast, cluster 2 showed increased autoantibody intensity in thymectomized children; these increased autoantibodies were enriched for the IgG isotype. After adjustment for multiple testing, the intensity of the following IgM autoantibodies was found to be significantly lower in thymectomized children compared to HC (Table 1): anti-ssDNA (single-stranded DNA), anti-C1q, anti-C4b, anti-ACE1 (angiotensin-converting enzyme 1), anti-LY96 (lymphocyte antigen 96), anti-Annexin 33Kda, anti-Thrombin, anti-p53, and anti-CD70. Concurrently, thymectomized children manifested significantly increased IgG autoantibody intensities toward cardiolipin and leptintA (leptin triple antagonist) (Table 1). To assess if these antibodies changes were due to neonatal Tx or that these autoantibodies were already present, we measured antibody reactivity in pre-Tx samples. The intensity of autoantibody reactivity increased after neonatal Tx when comparing to the pre-Tx plasma sample of the same child ($n = 4$, data not shown). The clinical consequences of these antibody repertoire alterations are not known as no clinical autoimmune disease was reported in young Tx.

Neonatal thymectomy does not affect B-cell compartment phenotype

The altered autoantibody profile post-Tx is likely due to changes in T cell help to B cells and this could be reflected in changes in different B-cells subsets. We therefore analyzed both B-cell differentiation and immunoglobulin production 1–5 years post-Tx. The proportion of CD19$^+$ B-cells in the total lymphocyte pool did not significantly differ between thymectomized children and healthy children. Further analyses of the immature/transitional, mature naïve, memory, and antibody-secreting stages of B cells showed no differences between the groups. CD21$^{-/low}$CD19^{high} and CD19$^+$CD5$^+$ B cells have been associated with autoimmune disease, but also for these subsets no differences in percentages were found (Table 2).

The level of total immunoglobulin and free light chains (FLCs) could shed further light on changes in B-cell function. Total IgM levels did not differ, but total IgG levels were significantly lower in the Tx group (Supporting Information Fig. 2A and 2B). No difference in the levels of kappa and lambda FLCs and their ratio were apparent between the groups (Supporting Information Fig. 2C).

Overall, neonatal Tx did not alter the B-cell subsets measured, but showed a decrease in total IgG levels.

Later in life, autoantibodies do not correlate with T-cell numbers or thymic function

We wondered if the altered autoantibody repertoires in thymectomized children might persist or even further develop later in life. We therefore examined ANA and ANCA autoantibodies in a cohort of adolescents and adults (older Tx) with an average follow-up of 16 years after neonatal Tx (9.1–29 years range). In this group, T-cell numbers were restored and ki-67 expression by CD4$^+$ T cells was similar to that in healthy controls (Fig. 4A and B). In the majority of older Tx individuals (16 of 26), autoantibodies against ANA and ANCA were detected: 14 were ANA-positive, 1 was ANCA positive, and 1 was positive for both ANCA and ANA. In contrast, in healthy age-matched controls only three of nine were autoantibody positive (ANA, $n = 3$) We further assessed antigen specificity in the ANA-positive Tx individuals, but no specific

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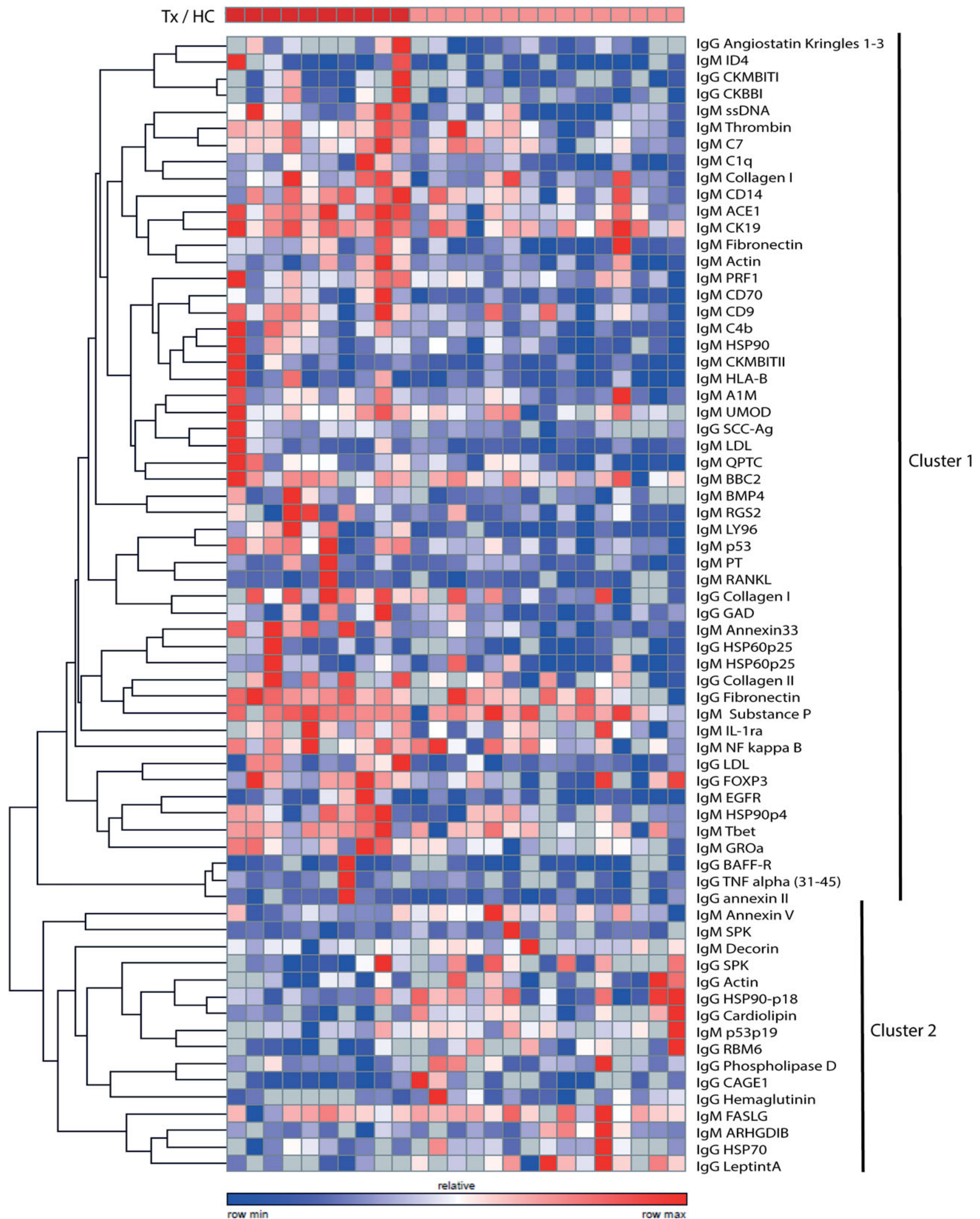

Figure 3. Thymectomized children manifest an altered autoantibody reactivity profile.
Plasma was isolated from heparinized blood samples and analyzed by antigen microarray. IgM and IgG self-antibody profile analysis of 68 differentially expressed antigens of HC ($n = 10$, red) and Tx ($n = 15$, pink). Data are representative of one experiment with 25 samples.

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Table 1. Antigen reactivity of the autoantibodies differentially expressed between thymectomized and healthy children

Antigen reactivity	Young HC	Young Tx	*p*-value
IgM ACE1	−0.02 (0.20)	−0.38 (0.19)	0.027
IgM LY96	1.67 (1.38)	−0.08 (0.37)	0.027
IgM Annexin33	0.21 (0.44)	−0.31 (0.22)	0.032
IgM Thrombin	−0.19 (0.12)	−0.42 (0.23)	0.032
IgM p53	0.16 (0.37)	−0.24 (0.21)	0.032
IgM DNAss	7.58 (3.67)	3.44 (2.62)	0.040
IgM CD70	0.56 (0.63)	−0.08 (0.27)	0.032
IgM C1q	−0.01 (0.27)	−0.28 (0.16)	0.027
IgM C4b	1.05 (0.93)	0.17 (0.43)	0.040
IgG LeptintA	−0.82 (0.06)	−0.59 (0.16)	0.027
IgG Cardiolipin	−0.78 (0.05)	−0.66 (0.08)	0.027

Results are corrected for multiple comparison (false discovery rate (FDR)), data shown as mean (SD).

Table 2. Phenotypical analysis of B-cell subsets, mean percentage (SD)

Phenotype	HC ($n = 7$)	Tx ($n = 11$)	*p*-value
$CD19^+$ B cell (within lymphocytes)	19.8 (10.2)	26.3 (5,6)	0.179
Immature/transitional (IgD^+CD27^-)			
T1, $CD38^{high}$ $CD10^+$	2.6 (0.9)	2.3 (0.7)	1.000
T2, $CD38^+$ $CD10^+$	1.7 (0.2)	1.8 (0.1)	0.930
T3, $CD38^+$ $CD10^-$	13.6 (2.4)	15.6 (2.4)	0.211
Mature naïve (IgD^+CD27^-)			
$CD38^-$ $CD10^-$	60.2 (7.1)	58.1 (10.2)	0.285
Memory B cell			
IgD^-CD27^-: double negative	5.4 (3.6)	5.5 (2.4)	0.596
IgD^+CD27^+: nonswitched	4.2 (2.0)	3.1 (1.4)	0.328
$IgD^-CD27^+IgM^+$: IgM memory	3.8 (3.0)	3.1 (1.8)	1.000
$IgD^-CD27^+IgM^-$: switched	1.0 (0.9)	0.9 (0.4)	0.536
Antibody secreting			
$IgD^-CD27^{high}CD38^{high}$	0.5 (0.6)	0.5 (0.3)	0.328
Autoreactive assoc. B cells			
$CD19^{high}CD21^{-/low}$	4.7 (2.6)	2.5 (1.0)	0.104
$CD19^+CD5^+$	41.1 (19.2)	31.1 (21.9)	0.710

reactivity toward nuclear antigens was found (nRNP, Sm, SS-A (SS-A native and Ro-52), SS-B, Scl-70, PM-Scl, Jo-1, CENP B, PCNA, dsDNA, nucleosomes, histones, ribosomal P-protein, and AMA M2). A T-cell clonal response with a possible subsequent skewed antibody response could also be due to prior cytomegalovirus (CMV) infection, but only five of 16 autoantibody positive Tx donors had CMV IgG antibodies (data not shown). Interestingly, in contrast to what we found in the young Tx group, the presence of autoantibodies in the older Tx group was not correlated with the percentage of memory T cells (Fig. 4C) or with the T-cell count (Fig. 4D).

In the majority of older Tx individuals, CD31 expression by naïve T cells normalized, suggesting that thymic tissue regenerated with thymic output (Fig. 4E). In seven of 26 patients, CD31 expression by naïve T cells was too low which suggested that regeneration of thymic tissue did not occur (Fig. 4E). The difference in CD31 expression by naïve $CD4^+$ T cells did not correlate with autoantibody alterations (Fig. 4E), while a lower CD4:CD8 ratio was still apparent in older children with low CD31 expression (Supporting Information Fig. 3). Together these data suggest that ANA positivity in the older neonatally thymectomized group did not correlate with the proportion of memory T cells or thymic tissue regeneration.

Preferential expansion of Treg cells in the lymphopenic phase following thymectomy

Peripheral tolerance by Treg cells could play an important role in preventing altered autoreactivity from becoming clinically overt autoimmune disease. Concomitant with the decrease in absolute $CD4^+$ T-cell numbers after neonatal Tx, a significant decrease in Treg cell numbers was observed, which normalized in later years (Fig. 5A). However, the *relative proportion* of Treg cells within the total $CD4^+$ T-cell compartment in the first years following Tx was significantly higher than in age-matched healthy controls (Fig. 5B). This increased proportion of Treg cells could be explained by the preferential proliferation of Treg cells compared to other $CD4^+$ T cells that is observed early after Tx (Fig. 5C). An increase in activated (aTreg, CD45RA-$Foxp3^+$) and cytokine secreting Treg cells (cTreg, CD45RA-$Foxp3^{dim}$) in the first years after neonatal Tx was apparent (Fig. 5D and Supporting Information Fig 4a). Later in life, no differences in the subpopulations of Treg cells were noticed between Tx individuals and healthy controls (Fig. 5D). We further assessed the suppressive function of Treg cells and did not find any differences between healthy and thymectomized individuals later in life (Supporting Information Fig. 4b). Also the stability of Foxp3, as measured by the demethylation status of the Treg cell specific demethylation region, did not differ between these two groups (Supporting Information Fig. 4c). Overall, a relative expansion of Treg cells was seen in the first years following neonatal Tx when T-cell lymphopenia was most evident. We observed no differences in the function and stability of Treg cells between Tx children and healthy controls.

Discussion

Neonatal Tx results, next to the loss of a lymphoid organ for T-cell education, in a temporary decrease in T-cell numbers and possibly increased rates of homeostatic T-cell proliferation [5, 6, 8]. We show here that neonatal Tx also results in qualitative changes in autoantibodies, which appears to correlate with increased peripheral T-cell expansion in early life. In addition, preferential expansion of Treg cells is seen, which might help to prevent the autoreactivity to become clinically overt.

Antigen microarray assays enable large-scale screening of hundreds of antibody reactivities involved in health and autoimmune diseases [28]. IgG autoantibodies have been associated with autoimmune disease, while IgM autoantibodies have been

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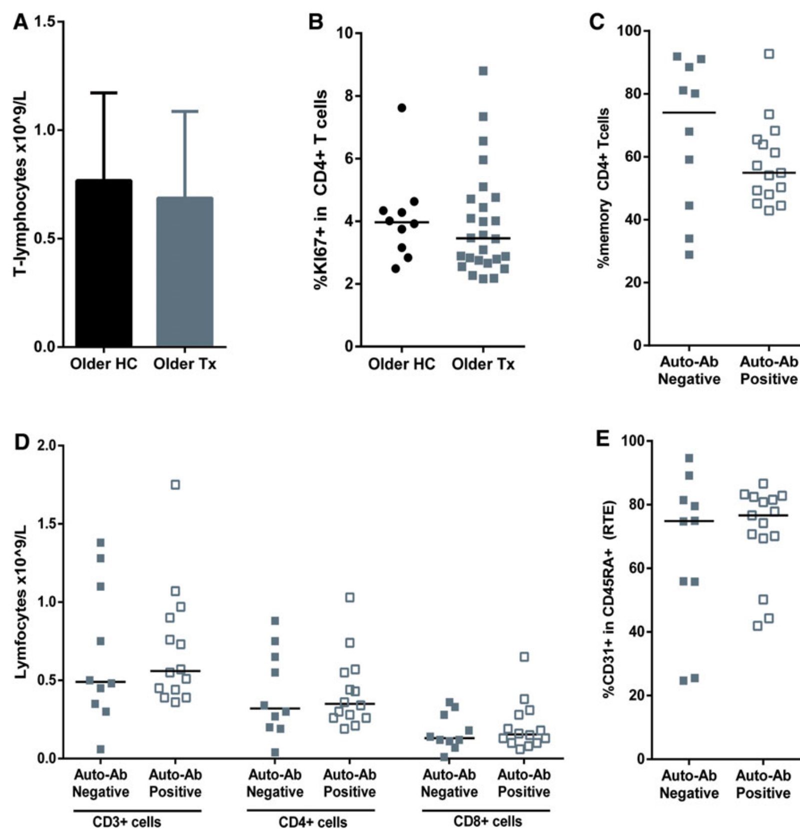

Figure 4. Autoantibodies persist later in life independent of T-cell number, thymic output, and memory CD4 T cells.
PBMCs and plasma were isolated from heparinized blood samples and analyzed by flow cytometry and autoantibody screening, respectively. (A) Lymphocyte count in healthy control (older HC, $n = 10$) and thymectomized (older Tx, $n = 24$) adolescents. Mean shown, error bars indicate SD. (B) Percentage of proliferation (ki-67+) in $CD4^+$ T cells of HC ($n = 10$) and Tx ($n = 26$). (C) Percentage memory ($CD45RO^+$) CD4 T cells in autoantibody positive (ANA and ANCA) and negative older Tx patients. (D) Lymphocyte count in autoantibody positive and negative Tx patients. (E) Recent thymic emigrants (RTE, $\%CD31^+$ in $CD45RA^+CD4^+$ T cells) in autoantibody positive ($n = 15$) and negative ($n = 10$) Tx patients. Panels A–E, data are pooled from five independent experiments with three to five samples per experiment; Panels B–E, median is shown. Statistical analysis: Mann–Whitney U test used.

associated with maintenance of self-tolerance [24, 25, 29]. The presence of IgM polyreactivity, for example, has been associated with reduced disease severity in lupus patients [30]. We observed increased IgG reactivity toward cardiolipin and leptin A after neonatal Tx. Anti-cardiolipin antibodies have previously been associated with several diseases, including antiphospholipid syndrome, rheumatoid arthritis and systemic sclerosis [31]. The adipocyte-derived hormone leptin is a pro-inflammatory cytokine that also has a potent role in mediating many autoimmune diseases [32, 33] In addition, we detected a decreased reactivity of multiple IgM natural autoantibodies in thymectomized children. Some of these IgM antibody reactivities were associated with antigens involved in systemic lupus erythematosus, such as C1q and DNAss [34]. This suggests that in the early years after neonatal Tx, maintenance of self-tolerance is disturbed, which may lead to increased IgG reactivity to self-antigens, and decreased IgM autoantibodies. In support of this, about 58% of neonatally thymectomized children manifested autoreactivity specifically toward ANA later in life, in comparison to 33% in the healthy control group. Although a relatively small group of healthy controls were assessed, the latter percentage is similar to what is reported in literature [35]. In any case, the alterations in the autoantibody repertoires that we observed in thymectomized children were not accompanied by overt autoimmune disease.

In several autoimmune diseases, the presence of autoantibodies is accompanied by changes in the Tfh and B-cell compartments [36–40]. Tfh cells are important to provide help to B cells to generate specific antibodies [41]. For several autoimmune diseases, increased proportions of Tfh cells and increased levels of CXCL3 and IL-21 have been documented; these have been proposed to lower the selection threshold for B cells and thereby to allow for the survival of low affinity or self-reactive B-cell clones [42, 43]. We here show that increased proportions of Tfh cells are also observed after neonatal Tx, as well as increased production of B lymphocyte chemoattractant (BLC, CXCL13). However, no drastic changes within the B-cell compartment were apparent after neonatal Tx; we found no evidence for loss of CD21 or increased CD5 expression on B cells, which have both been reported to be associated with autoimmune disease [21–23]. The production of FLCs is associated with disease activity in various autoimmune disorders including systemic lupus erythematosus and rheumatoid arthritis [44–46]. However, neither the level of kappa and lambda FLC, nor their ratio differed between neonatally thymectomized and healthy children. The only difference was a lower total IgG level in neonatally thymectomized individuals. This may be due to the temporarily lower number of T cells after neonatal Tx, which are necessary for Ig class switching. Together, it seems that the quantity of the B-cell responses measured are not affected by neonatal Tx, as no alterations in the

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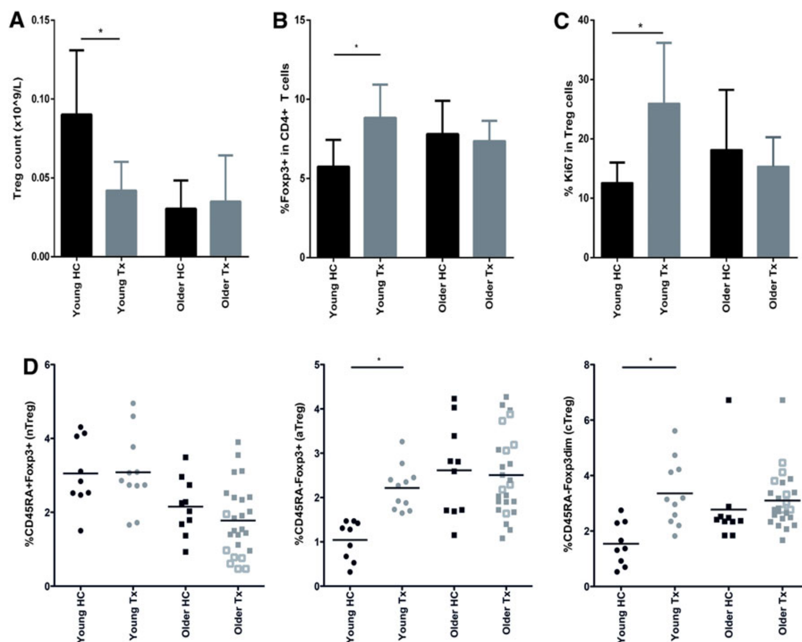

Figure 5. Preferential Treg-cell proliferation during the first years after Tx.
PBMCs were isolated from heparinized blood samples and analyzed by flow cytometry. (A) Treg cell ($CD4^+Foxp3^+$ T cells) count in "young HC" (n = 8), "young Tx" (n = 10), "older HC" (n = 10) and "older Tx" (n = 25) patients. (B) Percentage of Foxp3-positive cells in $CD4^+$ T cells in "young HC" (n = 9), "young Tx" (n = 11), "older HC" (n = 10), and "older Tx" (n = 25) patients. (C) Proliferation (%Ki-67^+) of Treg cells in "young HC" (n = 9), "young Tx" (n = 11), "older HC" (n = 10), and "older T" (n = 25) patients. (D) Percentage of $Foxp3^+CD45RA^+$ (nTreg, left panel), $Foxp3^+CD45RA^-$ (aTreg, middle panel), and $Foxp3^{dim}CD45RA^-$ (cTreg, right panel) in $CD4^+$ T cells of young children (HC 1–5 years, n = 9 black circles), young thymectomized (Tx 1–5 years, n = 11, gray circles), older children (Tx > 10 years, n = 11, black squares), and older thymectomized children (Tx > 10 years, n = 26, gray squares, and gray open squares for individuals who lack thymic regeneration). Panels A–D, data are pooled from three independent experiments with three to five samples per experiment. Mean shown, error bars indicate SD. Statistical analysis: Mann—Whitney U test. $^*p < 0.05$.

representation of the different B-cell subtypes and the global production of immunoglobulins and FLC were seen. Instead, neonatal Tx seems to affect the *quality* of the B-cell response and to skew it toward self-antigens.

Even though we detected a skewed autoantibody profile after neonatal Tx in early life, in line with previous observations there were no signs of clinical autoimmune disease [47]. Evaluation of specific autoantibodies after Tx was previously assessed, but none of these children had measurable ANA [48]. The percentage of memory T cells in the latter study also did not differ from that in healthy controls, while we found evidence for both the presence of autoantibodies and significantly higher proportions of memory $CD4^+$ T cells after neonatal Tx. This suggests that memory T-cell expansion may play a role in the generation of autoantibodies. In the study of Halnon and colleagues a higher titer of antibodies directed toward double-stranded DNA was found in thymectomized individuals with a low Thymic Recent Emigrant Circles (TREC) content in peripheral blood mononuclear cells, suggesting that increased autoreactivity correlates with decreased thymic output [49]. In a retrospective study of ANA-positive children, the height of the autoantibody titers also seemed to correlate with clinical disease. In this study of ANA positive individuals (cut-off used ≥ 1:40), 55% had a recognized autoimmune disease, but these children also had significantly higher ANA titers (≥1:160) than those with nonautoimmune etiologies (≤1:80). The ANA positive thymectomized patients in this report resembled the children without autoimmune disease, as they were weak positive at a titer of 1:100 [50]. In addition, we did not detect any specific nuclear antigen reactivity in autoantibody positive thymectomized children, in contrast to what is seen in autoimmune disease. The development of autoimmune disease is likely the result of failure in several regulatory factors that preserve an adequate homeostasis to self. Treg cells are known to be crucial in the maintenance of peripheral tolerance. A previous study showed preferential expansion of Treg cells after neonatal Tx, specifically of activated (aTreg) and cytokine secreting (cTreg) Treg cells [8], which we confirmed in the present cohort. In addition, we here show that the function and stability of these Treg cells does not differ from that in healthy controls later in life. It is tempting to hypothesize that the preferential proliferation of Treg cells after neonatal Tx suppresses the development of excessive autoreactivity in the lymphopenic environment, thereby preventing clinical autoimmune disease.

While neonatal Tx results in transiently absent thymopoiesis and thymic tissue function, in our study it also involves cardiac surgery. Cardiac surgery itself, without Tx, has been associated with appearance of autoantibodies, but these responses are usually transient [51, 52]. In addition, CMV infection is known to expand T cells and to skew them toward an oligoclonal repertoire, as is also the case following neonatal Tx [53]. These oligoclonal T cells could be a reason for altered B-cell reactivity due to skewed T-cell help. However, only five of 16 autoantibody-positive older Tx children were IgG positive for CMV. We now show an association between T-cell expansion and generation of autoantibodies in neonatally thymectomized individuals. Together this suggests that the altered autoantibody profile in these individuals is a consequence of the absence of the thymus and subsequent HP in the years after surgery, although we cannot exclude that it may have been fueled by acute trauma during surgery or CMV infection in some cases.

While human neonatal Tx does not seem to result in an increased incidence of autoimmune disease in the first decades

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of life in our cohort, in experimental models neonatal Tx has been shown to lead to severe organ-specific autoimmune disease that clinically differ per mouse strain [54]. Nevertheless, murine Tx leads to premature ANA production, increased proportions of memory T cells and Treg cells, similar to the observations in our cohort [55]. While there are obvious similarities between mouse and human Tx, the contribution of the thymus in T-cell homeostasis in both species differs remarkably. Naive T-cell homeostasis in mice is primarily dependent on thymopoiesis, while in human adults naive T-cell production is almost exclusively due to peripheral T-cell proliferation [3]. Extrapolation of experimental Tx findings to the human setting should therefore be performed with caution.

Overall, we show that neonatal Tx is associated with alterations in the autoantibody repertoire early in life. Surgery necessitating neonatal Tx has only been possible for about 30–35 years and autoantibodies can arise long before clinical symptoms develop [56]. It remains unknown whether alterations in autoreactivity in these thymectomized children will ever or prematurely develop into clinical autoimmune disease later in life, but our data suggest that these individuals may be at increased risk. We would therefore like to recommend minimal removal of thymic tissue during cardiac surgery and awareness of increased autoreactivity in this population.

Materials and methods

Patient selection and characterization

Patients who had undergone complete Tx during infancy because of surgery to treat congenital heart defect at the Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands, were included in this study. The age at which these patients were thymectomized was within the first month of life (8.4 ± 5.9 days, median ± SD). Blood samples were taken during follow-up at 1–5years (n = 10–27) and after approximately 10 years (n = 26) of neonatal Tx, due to previously shown possibility of thymic tissue regrowth after 5–10 years [6]. Exclusion criteria were clinical signs of infection at time of blood draw and the presence of a syndrome or genetic disorder (e.g., 22q11 deletion, trisomy 21). Clinical reports of all patients were available and were screened for the presence or indication of autoimmune disease at the time of blood draw.

A healthy control group, without major neonatal surgery, consisted of 1–5 year (n = 10–31) and >10 year (n = 11) old age-matched healthy children, who visited the University Medical Center Utrecht to undergo elective surgery and were considered immunologically healthy.

The study was approved by the medical ethical committee of the University Medical Center Utrecht (study number: 05–041K and 06–149K) and written consent was obtained from all study participants or their legal guardians in agreement with the revised Helsinki Declaration of 2008.

As cell counts and sufficient cells were not available for all samples due to the limitation in obtained blood amount taken from children, some data points are not shown for all study subjects.

Cell preparation and flow cytometry

PBMCs were isolated from heparinized blood samples by using the Ficoll Isopaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden), and viably frozen and stored in liquid nitrogen until further processing. The flow cytometry staining protocol is described elsewhere [57].

Antibodies against human CD5 (L17F12), CD8 (Sk-1), CD21 (B-Ly4), CD31 (WM59), CD38 (HIT2), KI-67 (B56), PD-1 (CD279, Clone MIH4), CXCR5 (RF8B2) were from BD Biosciences (San Jose, CA), Goat F(ab')2 IgM and IgG from Southern Biotech(Birmingham, AL), CD19 (J3-119) from Beckman Coulter (Fullerton, CA), CD10 (eBioCB-CALLA), Foxp3 (PCH101) from eBiosciences (San Diego, CA), CD25 (BC96), CD127 (HCD127) from Sony biotechnology, (San Jose, CA) and CD3 (UCHT1), CD4 (RPA-T4), CD27 (O323), CD45RO (UCHL1), CD45RA (HI100), IL-21 (3A3-N2) from Biolegend (San Diego, CA). Finally, stained mononuclear cells were washed twice in FACS buffer and run on an FACS Canto II and analyzed by using FlowJo software (Treestar). Naïve T cells were defined as $CD3^+CD45RA^+CCR7^+$, memory T cells as $CD3^+$ $CD45RO^+$, Treg cells as $CD3^+CD4^+Foxp3^+$, and Tfh as $CD3^+CD4^+CXCR5^+PD1^+$

Autoantibody measurement

Plasma samples from young thymectomized children (1–5years) were diluted 1:100 and incubated with HEp-20-10 cells and primate liver substrates for ANA analysis, 1:100 with sections of rat kidney for antimitochondrial autoantibodies, 1:100 with rat stomach for smooth muscle autoantibodies, 1:10 with formaldehyde and formalin-fixed neutrophils for detection of anti-neutrophil cytoplasmatic and perinuclear autoantibodies, 1:100 with primate stomach for detection of antigastric parietal cell autoantibodies, and 1:100 with smooth muscle cells from sections of primate oesophagus. After washing, attached antibodies were stained using a fluorescein-labeled antibody against human IgG. Two independent raters unaware of subject status evaluated nuclear staining and autoreactivity was rated negative (absent) or positive (weak or stronger staining). Plasma of ANA positive older Tx were tested with the ANA Profile 3 EUROLINE (EUROIMMUN, Lübeck), to 14 different antigens: nRNP, Sm, SS-A (SS-A native and Ro-52), SS-B, Scl-70, PM-Scl, Jo-1, CENP B, PCNA, dsDNA, nucleosomes, histones, ribosomal P-protein, and AMA M2.

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Antigen microarray

Antigen microarray chips were prepared as described elsewhere [26, 58, 59]. Briefly, 911 antigens, each at its optimal concentration, were spotted in tetraplicates on epoxy-activated glass substrates using a 48-pin robot (printed at ImmunArray Ltd., Israel, MicrogridII arrayer MG610, Genomics/Digilab). These antigens included proteins, synthetic peptides from the sequences of selected proteins, nucleotides, phospholipids, and other self and non-self molecules. The arrays were washed after blocking and incubated for 1 h at 37° with a 1:500 dilution of two detection antibodies, mixed together: a goat anti-human IgG Cy3-conjugated antibody, and a goat anti-human IgM Cy5-conjugated antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Image acquisition was performed by laser (Agilent Technologies, Santa Clara, CA) and the signal intensity was extracted using in house software. The quantitative range of signal intensity of binding to each antigen spot was 0.01–65 000; this range of detection made it possible to obtain reliable data at the 1:10 dilution of test samples. Problematic antigen microarray spots due to smudges or grainy texture were removed manually upon inspection. We then subtracted the background from the foreground for each of the test spot. Antigen reactivity was defined by the mean intensity of four replicates binding to that antigen on the microarray; antigen intensities with a mean value lower than zero were marked as missing values. Antigens with less than 70% nonmissing values from the total number of samples were removed from further analysis. Each chip was then normalized by its mean reactivity divided by the standard deviation. This was done to account for differences in total protein concentrations that affect the background intensity level. Results are corrected for multiple comparison (False discovery rate (FDR)).

Cytomegalovirus IgG detection

CMV serostatus was determined on selected plasma samples by an enzyme immunoassay (Enzygnost CMV/IgG, Siemens Healthcare Diagnostic Products, Marburg, Germany), according to the instructions of the manufacturer.

Regulatory T-cell suppression and demethylation assay

From healthy controls and older Tx, $CD3^+CD4^+CD25^+CD127^{low}$ Treg cells were sorted by flow cytometry on FACS Aria (BD Biosciences). PBMC were labeled with CellTrace Violet fluorescent dye (Invitrogen) to measure proliferation. Treg cells were cocultured with allogeneic healthy control PBMC (one donor) at a 1:2, 1:4, and 1:8 ratio and stimulated with plate bound anti-CD3 (eBiosciences, OKT3, 0.1μg/ml). At day 4, proliferation of $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry. Suppression (percentage) was calculated and compared to $CD4^+$ or $CD8^+$ proliferation without Treg coculture.

Treg cell specific demethylation region demethylation status was measured according to previously described method investigating 15 commonly investigated CpGs sites in flow cytometry sorted Treg cells [60].

Luminex

IL-21 and CXCL13 were measured in thawed plasma by multiplex technology (xMAP, Luminex, Austin, TX). The immunoassay was performed as described previously [61]. Aspecific heterophilic immunoglobulins were preabsorbed from all samples with heteroblock (Omega Biologicals, Bozeman MT). Acquisition was performed with the Biorad FlexMAP3D (Biorad laboratories, Hercules, CA) in combination with xPONENT software version 4.2 (Luminex). Data analysis was performed with Bioplex Manager 6.1.1 (Biorad).

Statistics

Statistical significance between two groups was assessed using the Mann–Whitney U test (MWU test) for unpaired data and Wilcoxon signed-rank test for paired data. Statistical difference is indicated with $^*p < 0.05$. Differentially reactive self-antigens were defined based on thresholds (p-value < 0.05, FDR < 0.2, T-test $>$ abs(1.5)). Unsupervised hierarchical clustering dendrogram analysis of the antigen microarray data was performed using sample distance metric of one minus pearson correlation. Microarray data were analyzed using R Statistical Software (Core Team R. R: A Language and Environment for Statistical Computing. Vienna; 2013.) and GeneE analysis platform [Gould J (2013). *GENE.E: Interact with GENE-E from R*. R package version 1.8.0, http://www.broadinstitute.org/cancer/software/GENE-E.

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Abbreviations: **ANA**: antinuclear antibody · **ANCA**: antineutrophil cytoplasmic antibody · **HP**: homeostatic proliferation · **Tfh**: follicular T helper cell · **Tx**: thymectomy

Full correspondence: Dr. Femke van Wijk, Laboratory of Translational Immunology (LTI), Wilhelmina Children's Hospital, University Medical Center Utrecht, Lundlaan 6, 3508 GA Utrecht
Fax: 0031–887553931
e-mail: F.vanwijk@umcutrecht.nl

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