

Letter to the Editor

Differential homeostatic dynamics of human regulatory T-cell subsets following neonatal thymectomy

To the Editor:

FOXP3-expressing CD4⁺ regulatory T (Treg) cells are important in the maintenance of self-tolerance and immune homeostasis. There are 2 possible origins for Treg cells:¹ (1) thymus-derived natural Treg (nTreg) cells and (2) peripherally induced Treg (iTreg) cells. Although both Treg cell populations express similar phenotypic proteins, it has been proposed that they exert different functions in maintaining immune homeostasis. While nTreg cells have been shown to be essential in self-tolerance, iTreg cells may be important in tolerance to nonpathogenic foreign antigens.² Disturbing the production of either Treg cell population may affect immune regulation later in life. Recently, it has been shown, for example, that alterations at neonatal age in thymic Treg cell maturation affects clinical outcome.³ It was demonstrated that in atopic children, thymic Treg cell function is significantly delayed early on in life. However, further data on human Treg cell development early in life are scarce.

The functional Treg cell population contains 2 distinct populations, a naive CD45RA⁺RO⁻FOXP3^{low} fraction and an activated/memory CD45RA⁻RO⁺FOXP3^{high} fraction, both equally capable of suppressive activity.⁴ Although both subpopulations

are true Treg cells, they have distinct differentiation dynamics. We hypothesized that Treg cell population dynamics would be affected in patients who undergo neonatal thymectomy during cardiac surgery. Previously, we showed the effect of neonatal thymectomy on long-term restoration of the naive T-cell compartment.⁵ In the present study, we evaluated the dynamics of distinct Treg cell subpopulations in the first 3 years following neonatal thymectomy.

Twenty-six children with a median age of 11.4 months (range, 2.5-34.7 months) who were previously thymectomized during the correction of a cardiac defect were included (see Table E1 in this article's Online Repository at www.jacionline.org). The study was approved by the medical ethical committee of the University Medical Center Utrecht (METC 05-041 and 06-149), and written informed consent was obtained. Thymectomy was performed within the first month of life (10.0 ± 9.0 days) in all participants. At the time of blood sampling, all children showed no sign of infection or immune dysregulation. For full information on the study population and flow cytometry staining protocols, see this article's Methods section in the Online Repository at www.jacionline.org.

First, we determined the impact of thymectomy on the total, peripheral CD4⁺ T-cell population. Following thymectomy, absolute CD4 counts dropped significantly compared with those in healthy infants (Fig 1, A; also see Fig E1, A, in this article's Online Repository at www.jacionline.org). Similarly, total FOXP3⁺CD4⁺ T-cell numbers were significantly lower in

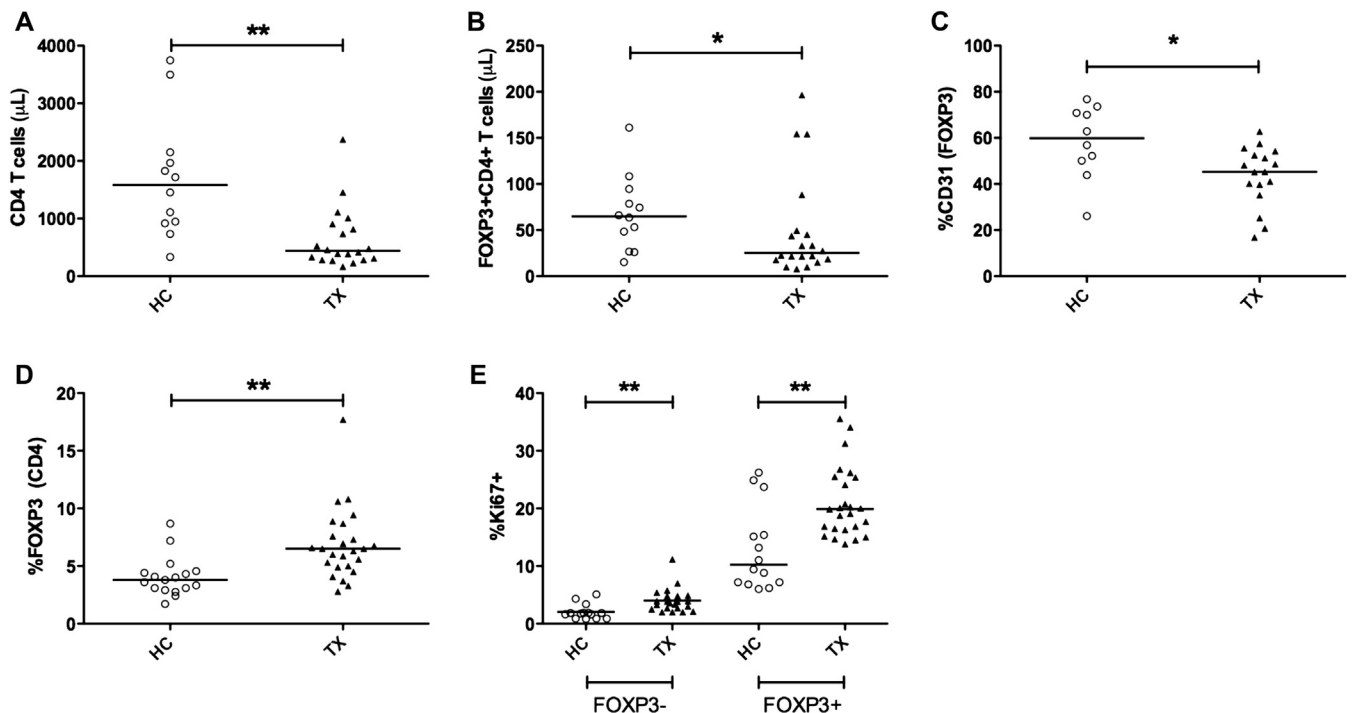


FIG 1. CD4⁺ and FOXP3⁺ T-cell dynamics and expression of CD31 and Ki67 after thymectomy. **A**, CD4⁺ T-cell count. **B**, FOXP3⁺CD4⁺ Treg-cell count. **C**, Percentage of CD31⁺ (FOXP3⁺) Treg cells. **D**, Percentage of FOXP3⁺ (CD4⁺) cells. **E**, Percentage of FOXP3⁻ and FOXP3⁺ T cells in cell cycle (Ki67⁺) in thymectomized subjects (TX) (▲) and age-matched controls (HC) (○). Horizontal line represents median value per group. * $P < .05$ and ** $P < .001$.

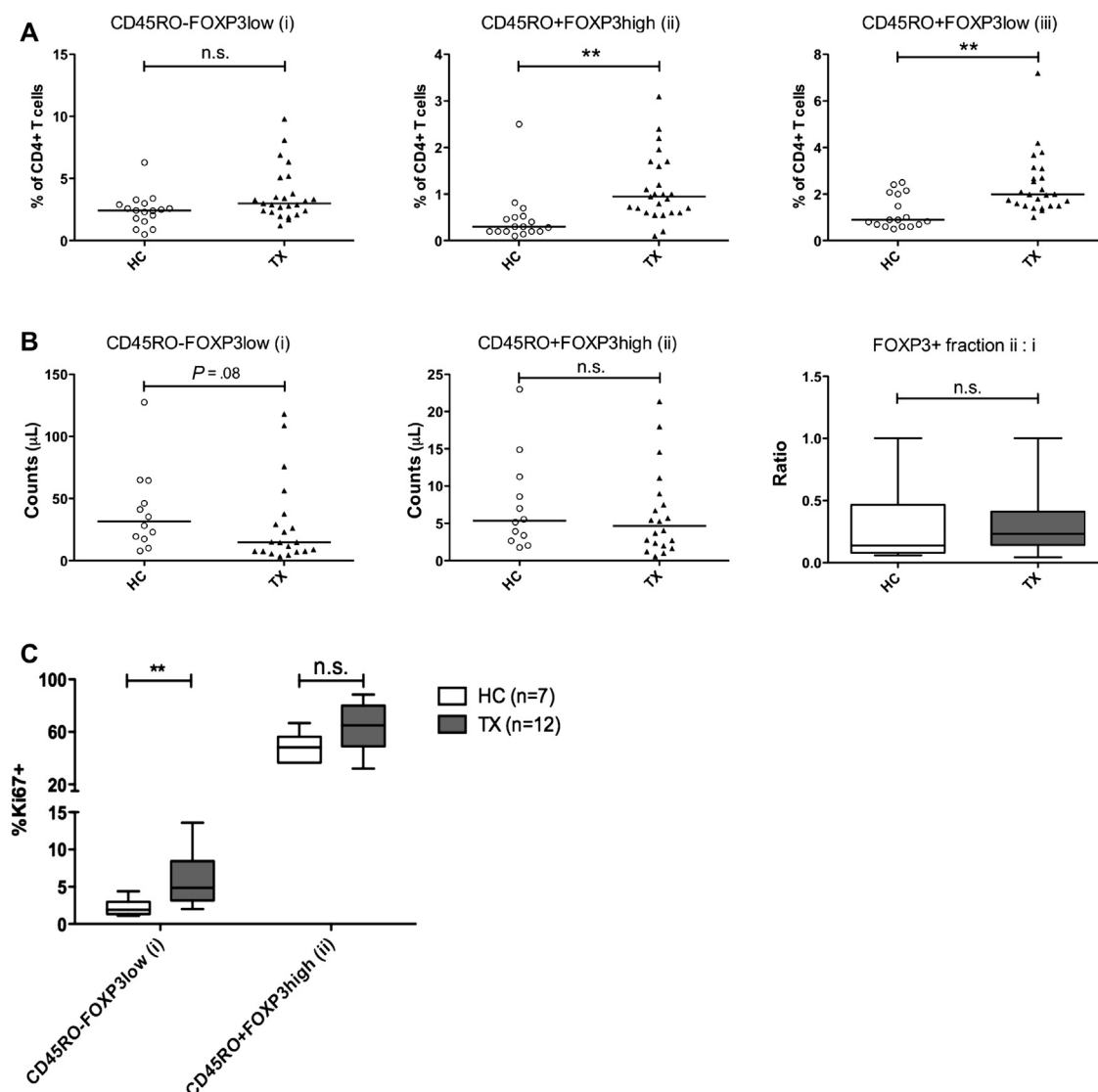


FIG 2. FOXP3⁺ subpopulation dynamics after thymectomy. **A**, Percentage of the 3 FOXP3⁺ subpopulations within CD4⁺ T-cell population. **B**, Cell count of the 2 FOXP3⁺ Treg-cell populations in healthy controls (HC) (○) and thymectomized patients (TX) (▲), and ratio of fraction ii:i. **C**, Expression of Ki67 per FOXP3⁺ Treg-cell population. n.s., No significant difference. **P* < .05 and ***P* < .001.

thymectomized patients than in healthy age-matched controls (Fig 1, B, and Fig E1, B). At a young age, CD31⁺ (PECAM-1) T cells represent recent thymic emigrants.⁶ Compatible with a loss of thymic production of Treg cells, thymectomized patients showed a significantly lower percentage of CD31⁺FOXP3⁺ T cells than did age-matched controls (Fig 1, C). Taken together, neonatal thymectomy results in a loss of thymus-derived Treg cells and a reduced number of circulating Treg cells.

Interestingly, after thymectomy, the percentage of FOXP3⁺CD4⁺ T cells was increased compared with that in controls (Fig 1, D, and Fig E1, C). Therefore, we investigated whether there was an increase in peripheral proliferation to compensate for the loss of thymic output. Compared with the FOXP3⁺CD4⁺ population, the FOXP3⁺CD4⁺ Treg cell population had a higher proportion of proliferation marker Ki67⁺ cells, with a significant increase following thymectomy (Fig 1, E). Thus, these data

suggest that there is a compensatory, selective expansion of Treg cells, leading to an increased percentage of Foxp3⁺CD4⁺ T cells.

Next, we studied the dynamics of 2 distinct Treg cell subpopulations; gating of the FOXP3⁺ fractions is illustrated in Figs E2 and E3 in this article's Online Repository at www.jacionline.org. The naive subpopulation CD45RO⁺FOXP3low (fraction I) and the memory population with the highest FOXP3 expression CD45RO⁺FOXP3high (fraction II) have been shown to be "true" Treg cells, whereas the memory population with low FOXP3 expression (fraction III) is nonsuppressive.⁴ We observed that the percentage of FOXP3 cells expressing the naive CD45 isoform (fraction I) remained stable, whereas both memory fractions increased significantly in thymectomized patients (Fig 2, A). The relative increase in the heterogeneous and nonsuppressive CD45RO⁺FOXP3low population (fraction III) may be a reflection of activation-induced transient upregulation of FOXP3, as

has been shown *in vitro*.⁷ Absolute numbers of both “true” Treg cell fractions decreased marginally, though not significantly (Fig 2, B). While numbers of CD45RO⁺FOXP3^{low} Treg cells (fraction I) were within the range of healthy controls, the majority of the patients had low numbers (Fig 2, B). Thus, neonatal thymectomy affects the composition of the Treg cell population.

When we examined the proliferation of the 2 “true” Treg cell subpopulations, a clear hierarchy was observed, with a 10-fold higher percentage of Ki67-expressing cells in CD45RO⁺FOXP3^{high} (fraction II) than in the naive CD45RO⁺FOXP3^{low} (fraction I) Treg cell population in both thymectomized and healthy individuals. Following thymectomy, an increase in proliferation of both subpopulations was found compared to the healthy controls, reaching statistical significance in the naive Treg cell fraction (fraction I) (Fig 2, C). Together, these data suggest that peripheral proliferation of both naive and activated Treg cells compensated for the loss of thymic output, resulting in maintenance of and increase in percentages of naive Treg and activated Treg cell populations, respectively (Fig 2, A). Although it is most likely that increased expansion of the activated Treg cell population is responsible for maintaining the number of activated Treg cells in these thymectomized children (Fig 2, B), we cannot exclude the possibility of additional increased conversion of naive Treg cells to activated CD45RO⁺FOXP3^{high} Treg cells.⁴ In a subgroup of patients, the number of naive CD45RO⁺FOXP3^{low} Treg cells was low despite increased proliferation, which appeared most prominent in the children older than 6 months (see Fig E4 in this article’s Online Repository at www.jacionline.org). A shift in the balance between naive and memory Treg cells has been associated with several pathological conditions. Reduced naive Treg cells with a compensatory increase in memory Treg cells has been associated with multiple sclerosis⁸ and sarcoidosis,⁴ while an increase in naive Treg cells, albeit with impaired suppressive function, has been observed in active systemic lupus erythematosus.^{4,9} Thus, removal of the thymus in the first month of life may affect immune regulation later in life. Overall, it is prudent to spare thymic tissue in patients requiring congenital heart surgery when technically possible.

This study demonstrates the specific homeostatic control of 2 distinct FOXP3⁺ Treg-cell populations. Peripheral proliferation of Treg cells counteracted the effect of loss of thymopoiesis, which illustrates the relative plasticity of the human immune system. However, changes in composition of the Treg cell population do warrant further investigation of the long-term

functional effects of neonatal thymectomy following cardiac surgery.

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METHODS

Study population and blood specimens

Twenty-six patients who all had undergone complete thymectomy at neonatal age during surgical correction for a congenital heart defect at the children's heart center, University Medical Center Utrecht, The Netherlands, were included in this study. The thymus is routinely removed during surgery involving the major vessels, such as transposition of the great arteries, hypoplastic heart syndrome, and hypoplastic aortic arch, because of its anatomical obstruction in relation to the heart. A healthy, age-matched, control group was included from both patients admitted for correction of a heart defect that did not necessitate removal of the thymus such as ventricular septum defects ($n = 9$) and healthy children who visited the University Medical Center Utrecht to undergo elective urologic or plastic surgery ($n = 8$). All included patients were considered immunologically healthy because they did not have a recent history of infectious disease or a hematologic or immunologic disorder. Patients with a known syndrome or genetic disorder were excluded (eg, 22q11 deletion and trisomy 21). Characteristics of the 26 included patients and healthy controls are depicted in [Table E1](#). Because cell counts were not available for all samples, absolute numbers of cell populations 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. Characterization of the T-cell compartment was performed on thawed cryopreserved PBMCs that were washed in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FCS and 0.1% sodium azide) and blocked with normal mouse and rat serum. The cells were incubated in 50 μ L FACS buffer containing the appropriately diluted antibodies against human CD3, CD4, CD45RO, and CD31. For intracellular staining of Ki-67 and FOXP3, the cells were first surface stained, followed by fixation and permeabilization according to the manufacturer's protocol. Antibodies against CD4 (clone SK3) and CD31 (WM59) were obtained from BD Biosciences (San Jose, Calif), against CD45RO (UCHL1) from Caltag (Buckingham, United Kingdom), against Ki67 (MIB-1) from Immunotech (Marseilles, France), and against FOXP3 (PCH101) from eBioscience (San Diego, Calif). Finally, stained mononuclear cells were washed twice in FACS buffer and run on an LSRII and analyzed by using FACSDiva software (BD Biosciences). The gates for the different populations were kept identical for each experiment containing both thymectomized patients and healthy controls.

Statistics

To analyze the quantitative differences between thymectomized patients and healthy age-matched controls, data only after thymectomy were included. Statistical significance between the 2 groups was assessed by using the Mann-Whitney U test for unpaired data and the χ^2 test for dichotomous data. Statistical difference is indicated as $*P < .05$ and $**P < .001$.

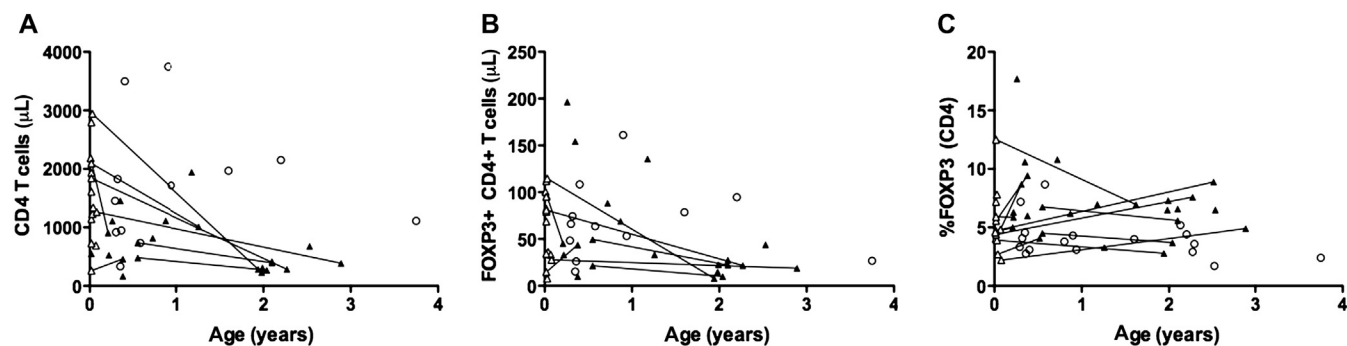


FIG E1. FOXP3 percentages and counts in the first years after neonatal thymectomy. **A**, Absolute CD4⁺ T-cell counts per microliter of blood. **B**, FOXP3⁺CD4⁺ Treg-cell counts per microliter of blood. **C**, Percentage of FOXP3⁺ cells in CD4⁺ T-cell populations. ▲, values after thymectomy; Δ, samples taken just before thymectomy; ○, healthy controls. Lines connect longitudinal samples.

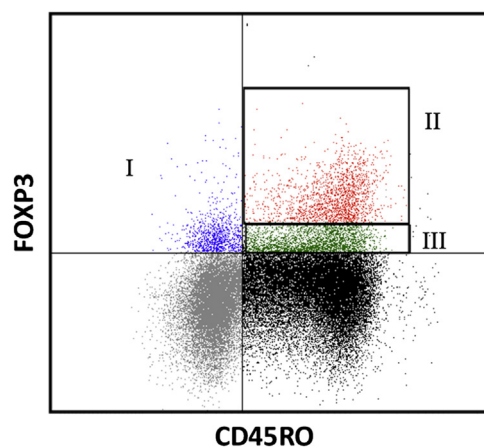


FIG E2. Gating strategy of subpopulations of FOXP3⁺ T cells. FOXP3⁺ subpopulations after gating for CD4⁺ lymphocytes. Fraction I, CD45RO⁻FOXP3^{low}; fraction II, CD45RO⁺FOXP3^{high}; fraction III, CD45RO⁺FOXP3^{low}.

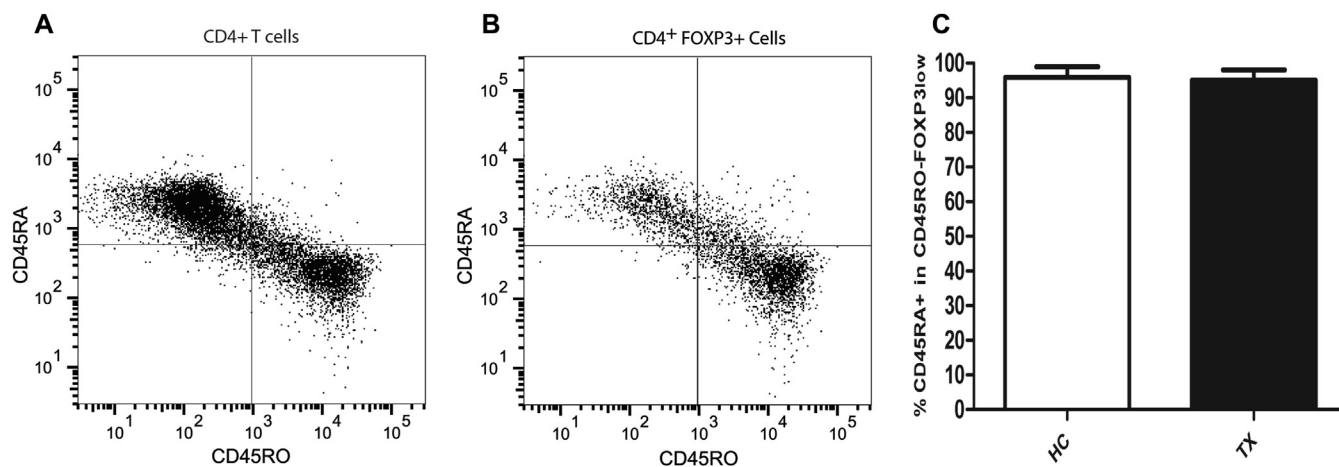


FIG E3. CD45RO⁺FOXP3⁺ T cells represent CD45RA⁺FOXP3⁺ T cells. **A**, Dot plot of CD45RO (memory) and CD45RA (naive) expression on CD3⁺CD4⁺ T cells. **B**, Dot plot of CD45RO and CD45RA expression on CD3⁺CD4⁺FOXP3 (Treg) cells. **C**, Expression of CD45RA⁺ in the CD45RO⁺FOXP3⁺ subpopulation. Healthy control group (HC), n = 7, and thymectomized patients (TX), n = 8. Data represented as mean percentage $\pm$ SD.

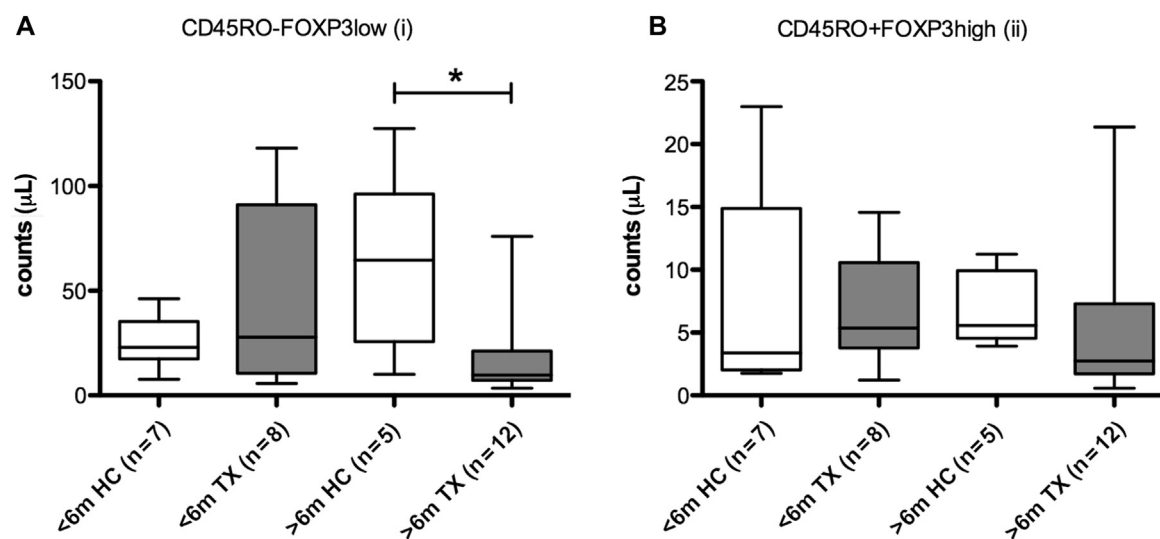


FIG E4. Subgroup analysis of naive and memory Treg-cell populations in 0- to 6-month-old and more than 6-month-old subjects. CD45RO⁻FOXP3^{low} naive Treg (**A**) and CD45RO⁺FOXP3^{high} memory Treg-cell (**B**) numbers in the subgroups less than 6 months and more than 6 months of age in healthy controls (HC) and thymectomized patients (TX). Data represented as median, 25% and 75% percentile boxes, and range.

* $P < .05$.

TABLE E1. Characteristics of the included patients and healthy controls

	Thymectomy	Control	<i>P</i> value
No. of subjects	26	17	
Age (mo)	11.4 ± 10.9	10.8 ± 12.6	.62
Age at TX (d)	10.0 ± 9.0	–	
Female:male	9:17	6:11	.96

TX, Thymectomy.