T cell phenotype in paediatric heart transplant recipients

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Abbreviations:

ALC - Absolute lymphocyte count
ATG - anti-thymocyte globulin
CHD - Congenital heart defect
CMV – Cytomegalovirus
EBV - Epstein Barr virus
MMF - Mycophenylate mofetil
MNCs - Mononuclear cells
NK - Natural killer
PTLD – post-transplant lymphoproliferative disorder
RTE - Recent thymic emigrant
TCR - T cell receptor
TREC - T cell recombination excision circle
Treg - T-regulatory cell

Abstract

Paediatric heart transplantation recipients suffer an increased incidence of infectious, autoimmune and allergic problems. The relative roles of thymus excision and immunosuppressive treatments in contributing to these sequelae are not clear. We compared the immunological phenotypes of 25 heart transplant recipients (Tx), 10 children who underwent thymic excision during non-transplantation cardiac surgery (TE) and 25 age-range matched controls, in two age bands: 1-9 and 10-16 years. Significant differences from controls were seen mainly in the younger age band with Tx showing lower CD3 and CD4 cell counts whilst TE showed lower CD8 cell counts. Naïve T cell and recent thymic emigrant proportions and counts were significantly lower than controls in both groups in the lower age band. T cell recombination excision circle levels (TREC) were lower than controls in both groups in both age bands. There were no differences in regulatory T cells, but in those undergoing thymic excision in infancy, their proportions were higher in TE than Tx, a possible direct effect of immunosuppression. T cell receptor V beta spectratyping showed fewer peaks in both groups than in controls (predominantly in the older age band). Thymus excision in infancy was associated with lower CD8 cell counts and higher proportions of Tregs in TE compared to Tx. These data are consistent with thymus excision, particularly in infancy, being the most important influence on immunological phenotype after heart transplantation.

1. Introduction

Thymic excision is often undertaken at the time of surgical correction of congenital heart defects (CHD) via median sternotomy. Such patients have been shown to have abnormal immunological findings in a number of studies, which have been reviewed[1,2]. Late clinical consequences in this group have been highlighted in a recent study[3]. Paediatric heart transplantation involves not only thymic excision but also peri-transplant immunosuppression and ongoing maintenance immunosuppression. Late graft loss/death in paediatric heart transplantation patients can be associated with an immunologically mediated coronary artery vasculopathy[4]. In addition, those patients transplanted in infancy suffer an increased incidence of allergic diseases, including eczema and asthma, and a variety of organ specific autoimmune disorders[5-7]. These problems may be due to immune dysregulation secondary to ongoing immunosuppressive treatment as well as the thymic excision. One previous study showed reduced naïve T cell production and reduced T cell diversity in twenty cases transplanted in infancy when compared to healthy controls but did not study a control group receiving thymic excision for correction of congenital heart disease without transplantation[8].

In order to ascertain the relative contributions of thymic excision and long-term immunosuppression on the immunological phenotype of paediatric heart transplantation cases, we set up a study to look at immunological phenotyping in these patients and compared the results to those in thymectomised, non-transplanted children and in non-thymectomised age-range matched controls.

2. Methods

2.1 Patients

Three groups of patients were recruited. This was performed by one of two clinicians approaching families attending for routine clinic follow up or for a procedure under general anaesthesia such as cardiac catheterisation on predetermined days when the laboratory was able to analyse the samples. Tx group comprised heart transplant recipients and TE group had undergone previous thymic excision at the time of correction of congenital heart disease. Patients with syndromes known to impact on thymic function including those with 22q.11 deletion, CHARGE syndrome and trisomy 21 were excluded. A control group of patients had a variety of different disorders not considered likely to affect immune phenotype and were attending the hospital on the same day. With informed consent of the parents and, where relevant, assent from the children, blood for analysis was obtained at the time of venesection for routine clinical purposes.

2.2 Laboratory Methods

Whole blood lymphocyte phenotype analysis was conducted by flow cytometry using a combination of directly conjugated monoclonal antibodies. Details of the antibody sources and panels used are shown in Table S1 (*Supporting Information*). Standard immunophenotyping included identifying B, T NK cells, CD4 T helper cells, CD8 cytotoxic T cells as well as memory, naïve and effector CD4 and CD8 T cells; these were performed in an ISO15189 accredited laboratory. The cell markers used in this study were those used in a previous study[9]. Naïve T cells were defined as those expressing CD45RA, CD27 and recent thymic emigrants (RTE) as those expressing CD45RA, CD31. Values were expressed as percentages of cells in the live gate and absolute numbers derived from the total lymphocyte count except in the case of naïve and RTE cells, which were derived from the absolute numbers of the relevant T cell subset (CD4/CD8). Since not all patients had a blood count measured at the time of venesection the numbers of data points obtained were less for absolute counts than percentages. Normal ranges for lymphocyte subsets and naïve populations were based on published data[10]. T cell recombination excision circles (TREC) levels were assessed on mononuclear cells (MNCs), isolated by density centrifugation, using real time quantitative polymerase chain reaction. Age related normal ranges for TREC were based on *in-house*, unpublished data, the 10^{th} percentile for 1-9 year olds being $10794/10^6$ T cells and for 10-16 year olds $5803/10^6$ T cells. Clonality of T cells was assessed using T cell receptor (TCR) V beta chain spectratyping on isolated CD3 positive cells as previously described[11]. To quantify these results, the average number of peaks per V beta family were counted[12].

Analysis of Treg populations was performed as described[28]. In brief, 1-2ml of whole blood was collected in EDTA tubes and processed within 4 hours for PBMC isolation using Lymphoprep density gradient centrifugation (Axis-shield, Norway). Details of the monoclonal antibodies used for characterization of Treg populations are provided in Table S1 (*Supporting Information*). Cells were stained in fluorescence-activated cell sorting (FACS) buffer (98%

PBS, 2% FCS, 0.001% Sodium Azide). Intracellular FoxP3 staining was performed in cells fixed and permeabilized according to the manufacturer's protocol (FoxP3 transcription factor staining buffer set, eBioscience, San Diego, CA). Samples were analysed immediately on LSRII using *FACSDiva* software (BD Bioscience). Results were analysed on *FlowJo* Data analysis software Version 9.1 (Ashland, Oregon). Single stain controls were run before each round of acquisitions using freshly isolated PBMC from healthy controls.

2.3 Statistics

For statistical analyses the data for Figures 1ACE, 2AC, 3ACE, and 4ABE and Supplementary Figures S1 and S2 were logarithmized and the significance of the differences between the three groups, *i.e.* controls (CTRL), heart transplantation (Tx), and thymectomy (TE), were estimated with R-package multcomp 1.4-12[27] using a one-way ANOVA approach for both cohorts independently, *i.e.* 1-9 years and 10-16 years, followed by Tukey all-pair comparisons between group means (post-hoc test). Data were $\log_2$ transformed (logarithmized) to get near normal distributions and are shown as Box-Whisker-Plots on linear scale with individual dots for each sample (the box represents the interquartile range (IQR), *i.e.* 25^{th} (Q1)-75^{th} (Q3) percentile, the horizontal line in the box the median, and the whiskers show the ranges of Q1-1.5*IQR and Q3+1.5*IQR, respectively). For Figures 1BDF, 2BD, 3BDF, and 4CDF a two-way ANOVA with two independent categorical variables, *i.e.* Tx versus TE and <1 Yrs versus >1 Yrs, was employed, again on logarithmized data. A two-way ANOVA allows the estimation of the impact of two independent categorical variables on one continuous dependent variable. P-values below 0.05 of the ANOVA tests were considered as statistically significant, and only if the ANOVA test was significant the post-hoc tests were considered. In the TCR V beta studies, the average number of peaks per family was shown to be non-normally distributed by the Shapiro Wilks test and so analysis was undertaken using the Mann Whitney U test.

2.4 Ethics

The collection of blood for the studies was conducted under approval from the Bloomsbury Research Ethics Committee, (formerly the Institute of Child Health and Great Ormond Street Hospital Research Ethics Committee).

3. Results

3.1 Patients

Tx patients. Twenty five heart transplant recipients were recruited. Details of these patients are shown in Table S2 (*Supporting Information*). Twenty (80%) of these patients had thymic excision at a separate procedure preceding heart transplantation either in the neonatal period for congenital heart defect in nine or to place a ventricular assist device in eleven. In terms of age at procedure, five (20%) had heart transplantation in infancy and a further seven (28%) had undergone thymic excision in infancy followed by heart transplantation at an older age. A total of 12 (48%) Tx patients therefore had thymic excision in infancy. Peri-transplant immunosuppression in 21 patients comprised two doses of the interleukin-2 receptor antagonist, Basiliximab, at the time of transplantation and then four days later followed by maintenance with tacrolimus and mycophenylate mofetil (MMF). The other four patients, who were transplanted at another centre, received anti-thymocyte globulin (ATG) with cyclosporine and azathioprine for the first year before switching to tacrolimus and MMF. At the time of testing, patients were receiving immunosuppression with: tacrolimus and MMF (16 patients); tacrolimus alone (8 patients); sirolimus, MMF and prednisolone (1 patient). None of the transplanted patients were considered to be in an acute phase of graft rejection at the time of testing.

TE patients. Ten patients were recruited of whom eight (80%) had undergone thymic excision in the first year of life, whilst two had later procedures at seven and eleven years respectively. From the established in-house practice and a review of the surgical notes, it was believed that all Tx and TE patients underwent complete removal of visible thymic tissue in the operative field, although we cannot exclude that some residual thymus tissue remained after surgery.

Control patients. Twenty-five control subjects were recruited. A list of their diagnoses is provided in *Supporting Information*. None were on immunosuppressive treatment. Six control subjects were venesected whilst under general anaesthesia. In view of the known age-related differences in lymphocyte numbers in children, patients and controls were grouped into two age bands for the purpose of analysis: 1-9 years and 10-16 years. Table S3 (*Supporting Information*) shows the ages of the patients and controls at the time of testing.

3.2 Clinical and virological status

As part of post-transplantation monitoring, all Tx patients underwent 26 regular testing by polymerase chain reaction (PCR) for viraemia caused by Epstein Barr virus (EBV) and cytomegalovirus (CMV). This was not undertaken in TE patients or in the control patient group. Fourteen Tx patients were EBV PCR positive at the time of the study and in a further five patients previous testing had shown EB viraemia on at least one occasion. Three patients had suffered post-transplant lymphoproliferative disease (PTLD) successfully treated with anti-CD20 monoclonal antibody (Rituximab) and reduction of immunosuppressive treatment. A further three had required reduction in

immunosuppression for high viral loads. None of the Tx patients had positive CMV PCR tests at the time of study but three had suffered previous CMV disease.

Clinical features consistent with immunodeficiency (infections, PTLD) and immune dysregulation (atopic and autoimmune phenomena) were found in 12/25 Tx patients and are summarised in Table S2 (*Supporting Information*). There were 2/10 TE patients with atopic symptoms but no other problems. Analysis of the data by age at thymus excision showed that eight of twelve Tx patients who had their thymus removed in infancy suffered these clinical problems whereas in the late TE patients, four of thirteen did so (Table S2, *Supporting information*).

3.3 Lymphocyte numbers

Data were analysed both by proportions of lymphoid cell types and absolute cell numbers as well as by age band and length of time after surgical procedure. Absolute lymphocyte counts (ALC) in both Tx and TE patients tended to be lower than controls but this was not statistically significant (ANOVA p=0.075 for the younger age band) (Figure 1A). T cell (CD3) and CD4 cell numbers were significantly lower than controls in the younger age band Tx patients (ANOVA p=0.017 and 0.008 respectively) (Figures 1C and 2A). CD8 cell numbers were lower in TE compared to controls in the younger age band (post-hoc test p=0.008) (Figure 2C). In contrast, NK cell counts were higher than controls in both Tx and TE patients at both age bands but significance was not reached (Figure 1E). In both groups, the proportions of T cells expressing the gamma/delta T cell receptor were not different from controls (*Supporting Information,* Figure S1).

Analysis by age at thymus excision: <1 v >1 year showed that there were no differences in Tx patients in this comparison and there were too few numbers in the >1 year age TE group to make such analysis possible. Comparing Tx and TE groups, CD8 cell counts were significantly lower in TE in those having thymus excision before 1 year of age (ANOVA p=0.007) (Figure 2D). In case this was a result of more EB virus infections in Tx patients, CD8 cell counts were compared between those with and without EB viraemia. CD8 counts tended to be higher in the EBV positive group but the difference was not statistically significant (median counts {range} 0.61{0.19-1.0}x10^6/ml in EBV positive and 0.38{0.22-0.97}x10^6/ml in EBV negative patients).

3.4 Naïve/Memory T cells, TREC and recent thymic emigrants (RTEs)

Naïve CD4 T cells (nCD4) expressed as counts were lower than controls in both groups in both age bands and these differences were significant for the younger age band (ANOVA p=0.0007, post-hoc tests: Control versus Tx and Control versus TE, p=0.003 and 0.001, respectively) (Figure 3A). A similar pattern was seen for naïve CD8 T cells (nCD8) for the younger age band. In addition, the TE older age band showed significantly lower nCD8 cell counts compared to controls (ANOVA p= 0.0008 and 0.021 for younger and older age band, respectively. Post-hoc test Control versus TE for older age band p=0.015) (Figure 3C). Expressed as percentages (*Supporting Information,* Figure S2A), nCD4 cells were

significantly lower than controls in both groups for the younger age band (post-hoc tests: Control versus Tx and Control versus TE, $p<0.0001$ and 0.024, respectively). In the 10-16 age band, significance was reached only for heart transplant recipients (post-hoc tests: Control versus Tx and Control versus TE, $p=0.03$ and 0.051, respectively). A significant difference in nCD8 cells was observed between control and Tx for both age bands (post-hoc tests: young and old age band, $p<0.001$ and 0.019 respectively) (*Supporting Information*, Figure S2B). A similar pattern was seen for proportions of RTEs with the exception that CD4 and CD8 RTEs in the Tx older age band were not significantly lower than in controls (*Supporting Information*, Figures 2C and 2D, respectively). TREC levels were markedly reduced compared to controls in both age bands (ANOVA tests: young and old age band, $p=0.00009$ and 0.0007, respectively) (Figure 3E). Two heart transplanted patients in the younger age band and one in the older age band showed TREC levels at or above the 10^{th} percentile of normal for age. One of the older age band TE patients also had a normal TREC level (Figure 3E). Ages at thymus excision for these particular patients were 3, 4 and 13 years for the Tx patients and seven months for the TE patient. In heart transplanted patients, there were no significant differences in naïve cells according to whether thymic excision took place before or after the age of one year (Figure 3F). nCD4 counts were however lower in TE than Tx patients undergoing thymic excision in infancy (Figure 3B). In the CD4 subset, non-naïve T cells were almost exclusively of classical memory phenotype (CD45RA-CD27+) in both groups and in controls. Only one TE patient showed elevated (>10%) proportions of effector cells at 11% of total CD4 cells. By contrast, in the CD8 subset 9/25 patients showed high proportions (>10%) of effector cells with a median of 23% (range 12-68%) and with low proportions of classical memory CD8 cells. Seven of these patients had current or previous EB viraemia, two had previous symptomatic 17 CMV infection and one had multiple autoimmune features. Only one TE patient had such an elevation at 34%. In the control group 7/25 patients also showed high proportions of effector cells with a median of 17% (range 16-23%). Virological results were not available in the controls but none of those with elevated effector CD8 cells had any history of problems with infections or autoimmunity.

3.5 Regulatory T cells

A representative dot plot showing the gating strategy for assessing Tregs is shown in *Supporting Information,* Figure S3. The proportions of these cells expressed as a percentage of CD4 cells and the absolute numbers were not significantly different between the two patient groups and controls but there was a trend for TE patients to have higher proportions of Tregs than Tx patients (Figure 4A and 4B). In patients undergoing thymus excision before one year of age, the TE group showed higher percentages of Treg compared to Tx patients but this was not significant (Figure 4C). This difference was not significant when the levels were expressed as absolute counts (Figure 4D). The proportion of cells expressing high levels of FoxP3 was highly variable but in neither group was significantly different from controls and did not vary significantly according to timing of thymus excision (Figure 4E and 4F).

3.6 TCR V beta spectratyping

Quantitative analysis of the spectratyping results showed a significantly higher average number of peaks per V beta family in the controls compared with either Tx (U=183, p=0.018)) or TE (U=41, p=0.007) patients (Figure 5A). Analysis of these data by patient age band showed that the reduction was only seen in those over 10 years of age whilst control subjects did not show any age-related variation (Figures 5B, C & D). There was no significant difference in the number of peaks between Tx and TE patients (U=68, p=0.17). In relation to timing of thymus excision, there were no significant difference Tx patients (Figure 5E). The numbers having thymus excision after one year were too few to make that analysis for TE patients.

4. Discussion

Our results show that both thymus excision in the context of corrective cardiac surgery for CHD, and in the context of heart transplantation with ongoing immunosuppressive treatment have broadly similar marked effects on the circulating T cell populations. These effects were most evident in cells bearing markers of naïve T cells, recent thymic emigrants and TREC. The reduction in counts was mainly seen in the younger patient age band. These findings are not unexpected in patients undergoing thymus excision and have been reported previously in studies after non–transplantation cardiac surgery[1,2,13,14] and in one similar sized study to this one in paediatric heart transplantation patients[8]. The present study confirms the previous findings after heart transplantation but in addition provides a comparison with non–transplantation, thymic excision cases. The study does show some subtle differences between the two groups. CD4 counts were lower than controls in the younger band Tx patients but not in TE patients. In fact, there was a trend to higher CD8 counts in Tx than TE patients in both age bands. Three Tx patients tested relatively early after their transplant had normal TREC levels, as did one of the TE patients tested late after thymus excision. Though the surgical procedures involved complete removal of 36 visible thymus tissue in all cases, it is possible that some, possibly ectopic, thymic tissue was not removed with subsequent thymic regrowth. It has been shown previously that thymic regrowth may occur in some patients after thymus excision[15]. TREC levels have been reported to be depressed after heart transplantation in adults (who do not generally undergo thymus excision) but, interestingly, were found to be present in higher proportions at the time of acute rejection episodes, which may have been due to effector T cells leaving the circulation with consequent enrichment of circulating naïve cells[16]. Apart from one case with chronic antibody mediated rejection, none of the transplanted patients was undergoing a rejection episode at the time of study.

The studies on effector/memory cell phenotype revealed that 9 (36%) of Tx patients had elevated proportions of effector CD8 cells in some cases to over 50% of the CD8 population. However surprisingly, 7 (21%) of the control group also had similar findings. High and persistent populations of effector cells have been reported to be driven by viral infections, such as CMV in both healthy and immunocompromised individuals[17, 18]. Two of the three Tx patients in our study with a history of CMV disease had such findings (Table S2). Seven of the nine had current or previous EB viraemia. There were no virological data available from the controls with this finding but none of the seven had a history of symptomatic infections or other immunopathological problems.

The proportions of CD4 cells with a Treg phenotype were not significantly different between either of the surgical groups and control children or between the groups themselves. These findings confirm those of others after thymus excision[19, 20]. Tregs are considered to be important for graft maintenance after solid organ transplantation, and in heart transplantation models they play a role in prevention of coronary artery vasculopathy[21, 22]. There are several ongoing clinical studies of use of adoptive transfer of Tregs to help induce tolerance after solid organ transplantation [23]. Function of Tregs was not tested in our study, but has been shown to be maintained in thymectomised patients[20] and adult heart transplantation patients, though

reduced function of these cells was found during acute rejection episodes[24]. Studies of Treg function after paediatric heart transplantation and after thymus excision without transplantation would help elucidate the role of these cells in graft maintenance and in immunopathological complications.

The tendency for the numbers of T cell subsets to be more closely matched to controls in the older age group could be explained by homeostatic expansion of memory T cells. In keeping with this, TCR V beta spectratyping showed less variability in the older age bands in both Tx and TE patients. Abnormal V beta usage after thymus excision and heart transplantation has been previously shown but the time related changes after thymus excision were not highlighted[8,19]. The discrepancy between CD8 cell numbers in the two groups suggests that homeostatic proliferation does not seem to have occurred in the CD8 population of TE patients. CD4 and CD8 cells have been shown to have distinct cytokine requirements for this process[25]. It is possible that events such as viral infections or low-grade alloreactivity resulted in CD8 cell proliferation in Tx but not TE patients. There was a high rate of EB virus positivity in Tx patients and those who were positive did have higher CD8 counts than those who were negative though this did not show statistical significance.

Previous studies showed that allergic and autoimmune disorders were prevalent in paediatric heart transplant patients who underwent their procedure in infancy[5-8], and that allergic disorders reflected a Th2 shift that might protect against Th1 processes that mediate graft rejection[7]. We therefore compared those patients who underwent their surgical procedure before 1 year of age with those who were older than 1 at the time of surgery within each group. In Tx patients, there was indeed a trend for those who underwent thymus excision in infancy to have more clinical issues consistent with immune dysregulation than those undergoing thymus excision later. A similar comparison was not possible in TE because very few had undergone thymic excision beyond the neonatal period. However, in patients undergoing thymus excision in infancy, CD8 counts were lower and the proportion of CD4 T cells expressing a Treg phenotype was higher in TE compared to Tx patients. The lower Treg proportions might reflect a direct effect of ongoing immunosuppression from an early age.

There are weaknesses in this study. With banding into different age groups, relatively small numbers were present for analysis. Further studies with larger numbers would help confirm these findings. Furthermore, our heart transplantation cohort has a relatively high incidence of EB virus infections that might have had some influence on the parameters measured. This is a reflection of the necessity for using adult and older teenage donors. The high rate of PTLD in the cohort studied was not a reflection of the average rate in the unit, which is more in line with other centres at around 5-6%.

A recent birth cohort study showed that early thymectomy for correction of CHD significantly increased the risk of individuals developing infective, autoimmune and malignant disorders later in life[3]. Thus, where possible, total thymus excision should be avoided at the time of paediatric cardiac surgery. Whilst the data presented here and the findings of others are consistent with the same risks being present in heart transplantation patients, their situation is

more complex because thymectomy-mediated immune suppression may provide some protection against graft rejection later in life.

In conclusion, these data are consistent with T cell abnormalities in paediatric heart transplant recipients being predominantly a consequence of the thymic excision undertaken either prior to or as part of the procedure. When thymic excision is undertaken in infancy there are more clinical consequences than at older ages as shown by previous studies.

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6. Author Contributions

KM: Laboratory analyses, data analysis and statistics, drafting of manuscript
FK: Patient and controls recruitment, data collection and analysis
NS: Laboratory analyses
EW: Laboratory analyses, data analysis and statistics
SR: Laboratory analyses
C-IL: Laboratory analyses
SA: Data interpretation and statistics, critical revision of manuscript
KG: Data interpretation, critical revision of manuscript
DP: Data analysis and statistics, critical revision of manuscript
TC: Concept, design and funding of study, data interpretation, critical revision of manuscript
MB: Concept, design and funding of study, patient recruitment, critical revision of manuscript
EGD: Concept, design and funding of study, data interpretation, drafting of manuscript

7. Disclosures

The authors of this manuscript have no conflicts of interest to disclose.

8. Data Availability

The data that support the findings in this study are stored confidentially within the firewall of the UK National Health Service. De-identified raw data can be made available by the corresponding author upon reasonable request.

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Mengrelis K, Kucera F, Shahid N et al.2019. Immunophenotyping in heart transplant recipients. Stored within firewall of UK National Health Service.

Legends to Figures

Figure 1: Absolute lymphocyte count and T and NK cell counts by age band in Heart Transplantation (Tx), Thymectomy (TE) and age-range matched control subjects and by age (<1 versus >1 year) at thymus excision. yo – years old. All counts are $x10^6$/ml. (A): absolute lymphocyte count. (B): absolute lymphocyte count by age at thymus excision. (C): CD3 counts. (D): CD3 counts by age at thymus excision. (E): NK cell counts. (F): NK cell counts by age at thymus excision. The significance of the differences between groups (control subjects, heart transplantation and thymectomy) in both cohorts (1-9yo and 10-16yo) was estimated with ANOVA and post-hoc tests on log_2 transformed data. The significance of the differences between both independent grouping variables (Tx versus TE and <1 versus. >1 year) was estimated with a two-way ANOVA on log_2 transformed data. * $p < 0.05$

Figure 2: CD4 and CD8 positive T cells by age band in Heart Transplantation (Tx), Thymectomy (TE) and age-range matched control subjects and by age (<1 versus >1year) at thymus excision. yo – years old. All counts are $x10^6$/ml. (A):CD4 counts (B):CD4 counts by age at thymus excision. (C): CD8 counts. (D): CD8 counts by age at thymus excision. The significance of the differences between groups (control subjects, heart transplantation and thymectomy) in both cohorts (1-9yo and 10-16yo) was estimated with ANOVA and post-hoc tests on log_2 transformed data. The significance of the differences between both independent grouping variables (Tx versus TE and <1 versus. >1 year) was estimated with a two-way ANOVA on log_2 transformed data. * $p < 0.05$; ** $p < 0.01$

Figure 3: Naïve T cells, recent thymic emigrants and TRECs by age band in Heart Transplantation (Tx), Thymectomy (TE) and age-range matched control subjects and by age (<1 versus >1year) at thymus excision. yo – years old. (A): CD4+CD45RA+CD27+ (naïve CD4) counts. (B): CD4+CD45RA+CD27+ (naïve CD4) counts by age at thymic excision. (C): CD8+CD45RA+CD27+ (naïve CD8) counts. (D): CD8+CD45RA+CD27+ (naïve CD8) counts by age at thymic excision. (E): T cell recombination excision circle (TREC) levels determined on total mononuclear cells (MNCs). (F): T cell recombination excision circle (TREC) levels determined on total MNCs. The significance of the differences between groups (control subjects, heart transplantation and thymectomy) in both cohorts (1-9yo and 10-16yo) was estimated with ANOVA and post-hoc tests on log_2 transformed data. The significance of the differences between both independent grouping variables (Tx versus TE and <1 versus. >1 year) was estimated with a two-way ANOVA on log_2 transformed data. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 4: Regulatory T cells (Tregs) by age band in Heart Transplantation (Tx), Thymectomy (TE) and age-range matched control subjects. mo – months. yo – years old. Counts are $x10^4$/ml. Percentages are of CD4+T cells. (A): Percentage of CD25+/CD127low (Treg). (B): Treg

counts. (C): Treg percentages in relation to age at thymic excision (D): Treg cell counts in relation to age at thymic excision (E): Percentage of Treg phenotype cells expressing intracellular FoxP3. (F): Percentage of Treg phenotype cells expressing intracellular FoxP3 in relation to age at thymic excision. The significance of the differences between groups (control subjects, heart transplantation and thymectomy) in both cohorts (1-9yo and 10-16yo) was estimated with ANOVA and post-hoc tests on log_2 transformed data. The significance of the differences between both independent grouping variables (Tx versus TE and <1 versus. >1 year) was estimated with a two-way ANOVA on log_2 transformed data.

Figure 5: T cell receptor V beta spectratyping data analysed to calculate the average number of peaks per V beta subfamily in Heart Transplantation (Tx), Thymectomy (TE) and age-range matched controls. (A): data from all age groups combined. (B, C & D): younger (1-9 years) versus older (10-16 years) patients and controls. (E): data split by age at thymus excision <1 year versus > 1 year compared to controls without thymus excision.
Note: data available on insufficient to plot TE >1year.

Figure 1

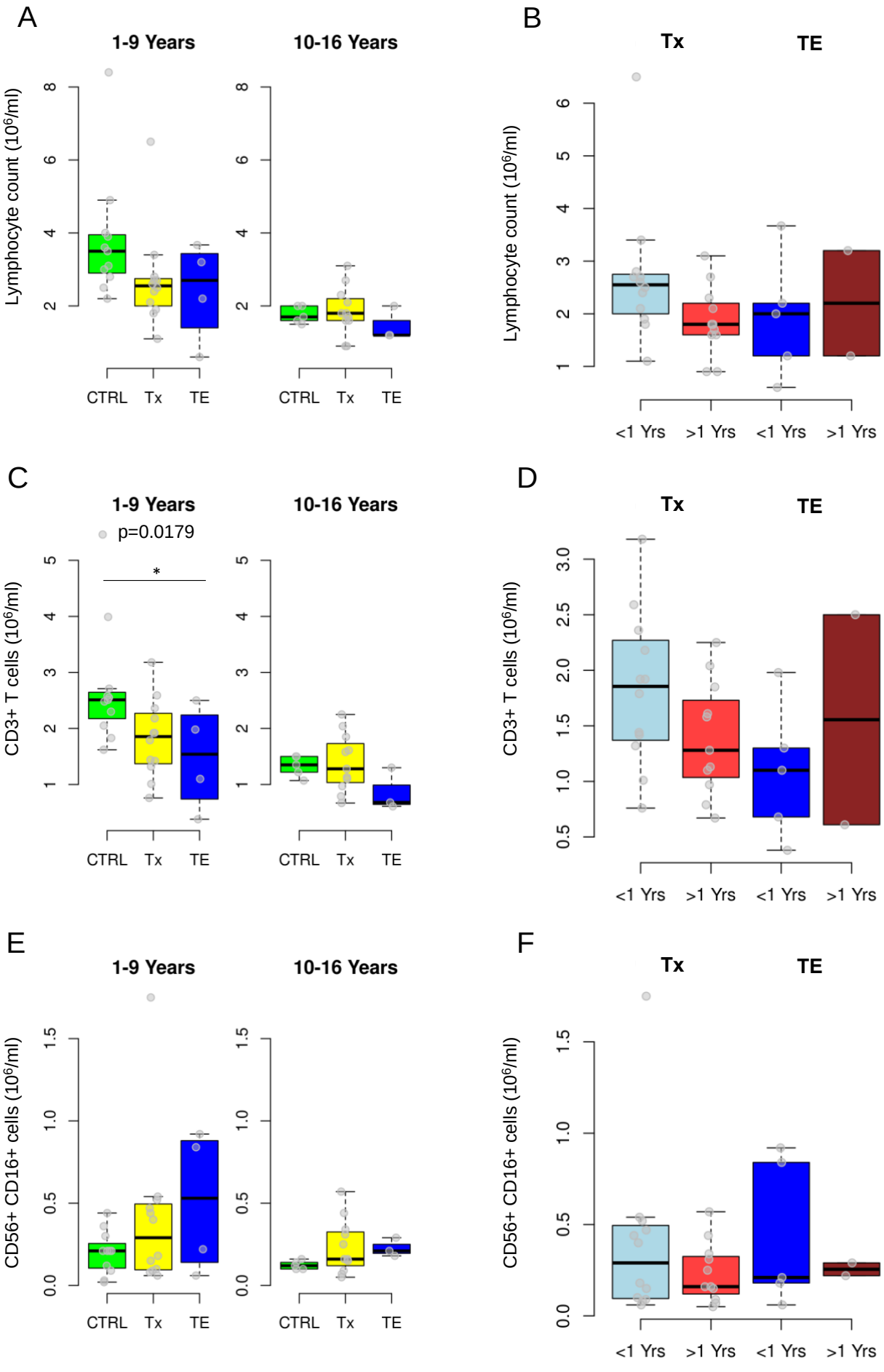

A
1-9 Years
10-16 Years
Lymphocyte count (10⁶/ml)
CTRL
Tx
TE
B
Tx
TE
Lymphocyte count (10⁶/ml)
<1 Yrs
>1 Yrs
C
1-9 Years
10-16 Years
p=0.0179
*
CD3+ T cells (10⁶/ml)
D
Tx
TE
CD3+ T cells (10⁶/ml)
E
1-9 Years
10-16 Years
CD56+ CD16+ cells (10⁶/ml)
F
Tx
TE
CD56+ CD16+ cells (10⁶/ml)

Figure 2

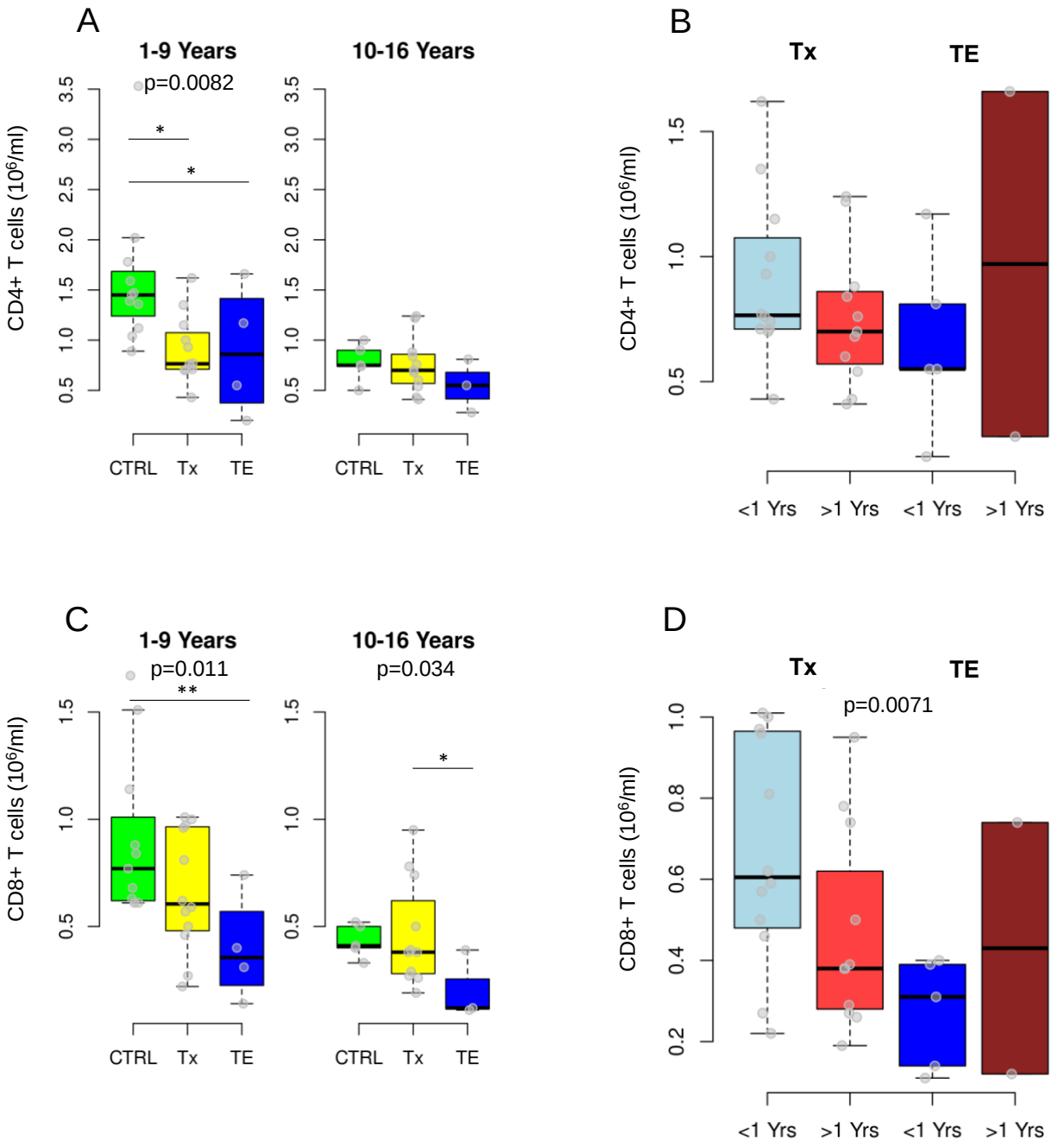

A
1-9 Years
10-16 Years
p=0.0082
*
*
CD4+ T cells (10⁶/ml)
0.5
1.0
1.5
2.0
2.5
3.0
3.5
CTRL
Tx
TE
B
Tx
TE
CD4+ T cells (10⁶/ml)
<1 Yrs
>1 Yrs
<1 Yrs
>1 Yrs
C
1-9 Years
10-16 Years
p=0.011
p=0.034
**
*
CD8+ T cells (10⁶/ml)
D
Tx
TE
p=0.0071
CD8+ T cells (10⁶/ml)
0.2
0.4
0.6
0.8
1.0

Figure 3

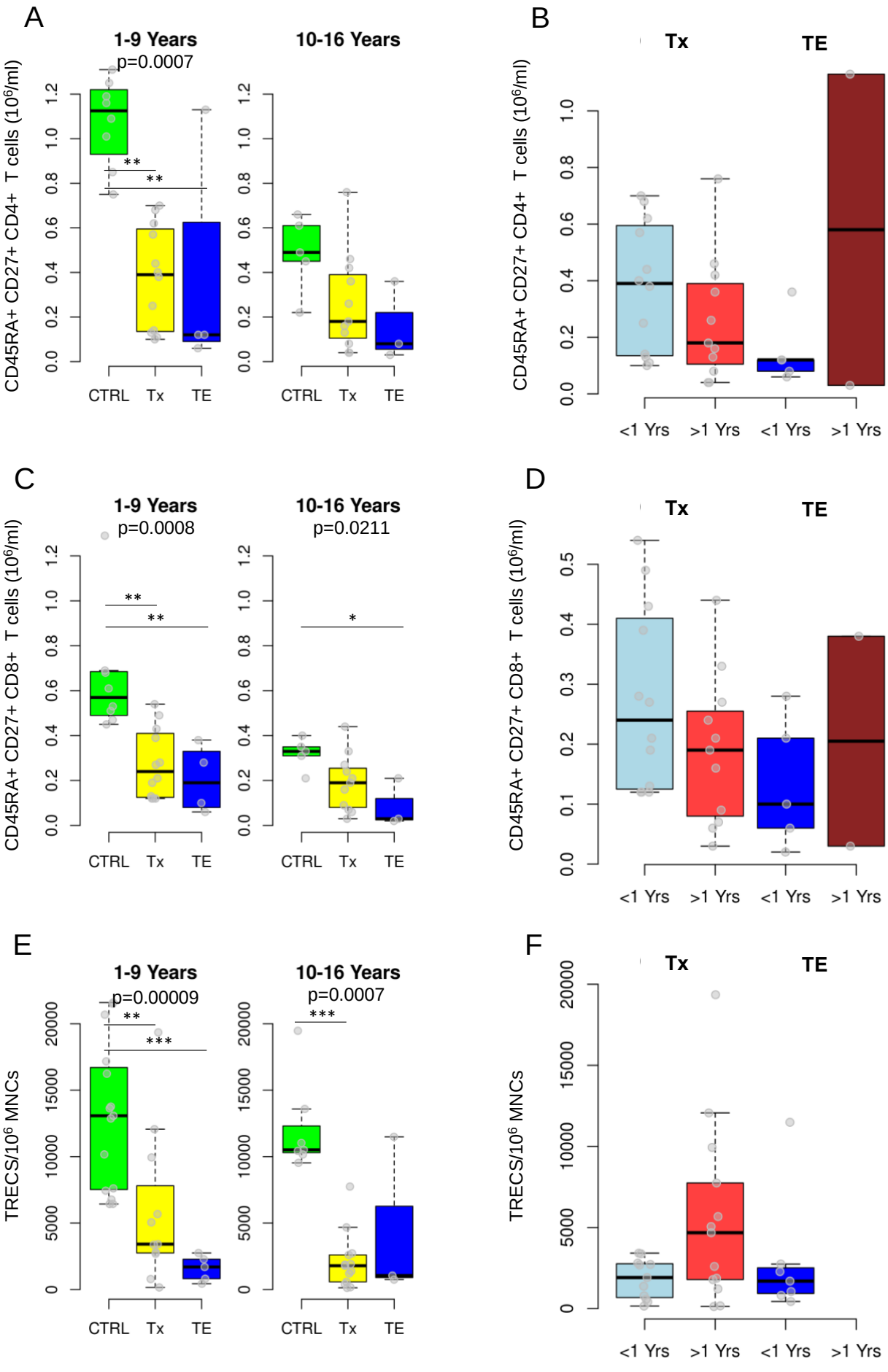

A
1-9 Years
p=0.0007
10-16 Years
CD45RA+ CD27+ CD4+ T cells (10⁶/ml)
CTRL
Tx
TE
B
Tx
TE
<1 Yrs
>1 Yrs
C
1-9 Years
p=0.0008
10-16 Years
p=0.0211
CD45RA+ CD27+ CD8+ T cells (10⁶/ml)
D
E
1-9 Years
p=0.00009
10-16 Years
p=0.0007
TRECS/10⁶ MNCs
F

Figure 4

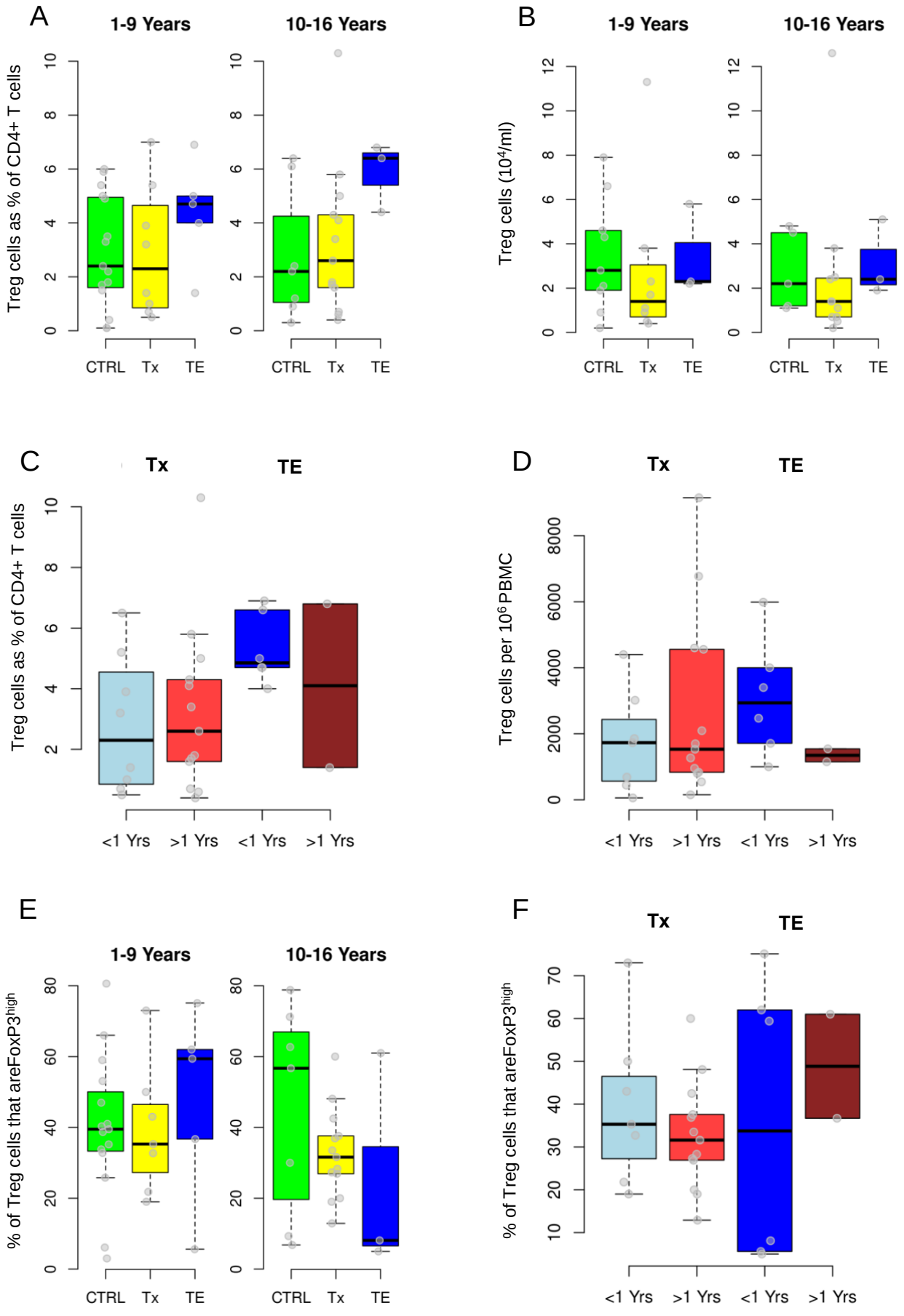
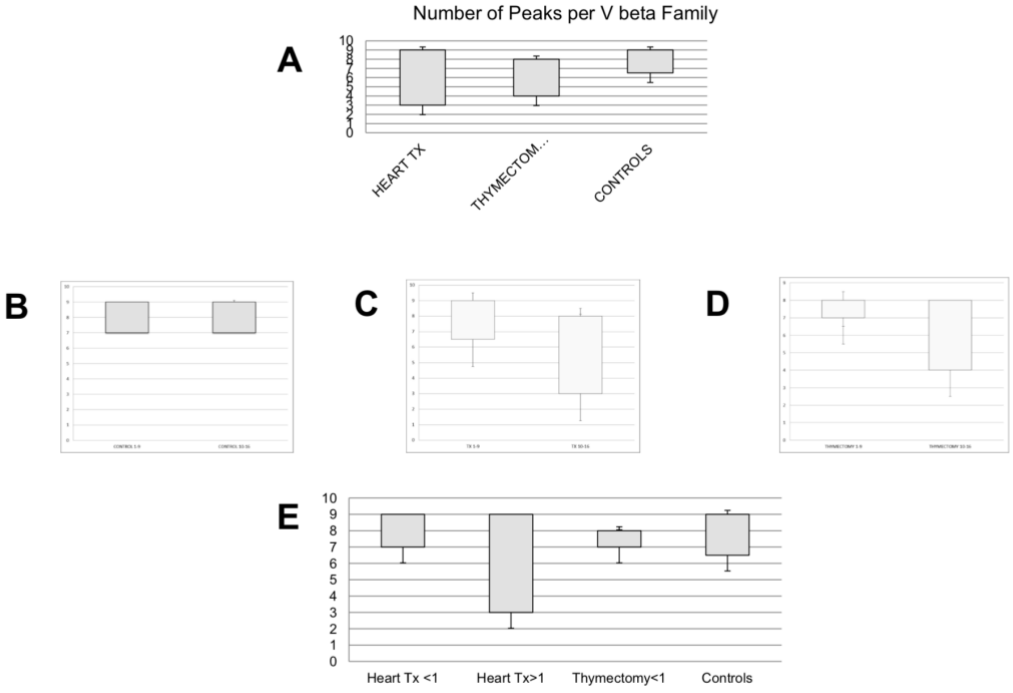

A
1-9 Years
10-16 Years
Treg cells as % of CD4+ T cells
CTRL
Tx
TE
B
1-9 Years
10-16 Years
Treg cells (10⁴/ml)
CTRL
Tx
TE
C
Tx
TE
Treg cells as % of CD4+ T cells
<1 Yrs
>1 Yrs
<1 Yrs
>1 Yrs
D
Tx
TE
Treg cells per 10⁶ PBMC
<1 Yrs
>1 Yrs
<1 Yrs
>1 Yrs
E
1-9 Years
10-16 Years
% of Treg cells that areFoxP3high
CTRL
Tx
TE
F
Tx
TE
% of Treg cells that areFoxP3high
<1 Yrs
>1 Yrs
<1 Yrs
>1 Yrs

Figure 5


A
Number of Peaks per V beta Family
HEART TX
THYMECTOM...
CONTROLS
B
C
D
E
Heart Tx <1
Heart Tx>1
Thymectomy<1
Controls

Supporting information

T cell phenotype in paediatric heart transplant recipients

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Control Group Details

Control patients were recruited at the time of attendance for outpatient consultation. Their diagnoses comprised: congenital heart defect not corrected through median sternotomy (7); genetically determined cardiomyopathy (6); Duchenne muscular dystrophy, not on corticosteroids (3); arrhythmia (2); developmental delay (2) and one each of: craniosynostosis, familial exudative vitreopathy, primary pulmonary hypertension, arginosuccinic acid lysase deficiency, anthracycline induced cardiomyopathy (six years after treatment for osteosarcoma).

Legends to Figures

Figure S1: Percentages of T cell subsets in Heart Transplantation (Tx) and Thymectomy (TE) patients. (A): CD3+T (B): CD4+ (C): CD8+ (D): CD16+CD56+ (NK) cells. (E): CD3+ cells expressing gamma/delta T cell receptor. The significance of the differences between groups (control subjects, Tx and TE) in both cohorts (1-9yo and 10-16yo) was estimated with ANOVA and post-hoc tests on $\log_2$ transformed data. ** $p < 0.01$

Figure S2: Percentages of naïve T cells and recent thymic emigrants in Heart Transplantation (Tx) and Thymectomy (TE) patients. Percentages are of CD4 or CD8+ T cells. (A): CD4+CD45RA+CD27+ (naïve CD4) (B): CD8+CD45RA+CD27+ (naïve CD8) (C): CD4+CD45RA+CD31+ (CD4 RTE) (D): CD8+CD45RA+CD31+ (CD8 RTE). The significance of the differences between groups (control subjects, heart transplantation and thymectomy) in both cohorts (1-9yo and 10-16yo) was estimated with ANOVA and post-hoc tests on $\log_2$ transformed data. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$

Figure S3: Representative dot plots depict flow cytometric gating strategy for enumerating Treg populations. SSC - side scatter. FSC - forward scatter

Table S1. Antibody panels used in flow cytometric analyses

Panels	Antibodies
LSS (Lymphocyte subsets)	**CD3/CD16+56/CD45/CD19/CD4/CD8 (*BD* 644611)**
LSS + T Memory	**[3/16+56/45/4/19/8] *(BD* 644611)** **CD45RA V450 (*BD* 560362)** **CD27 V500 (*BD* 561222)**
TCT (T Cell Types)	**[αβ/γδ/CD3] (*BD* 335836)** **CD8 APC (*BD* 345775)** **CD4 APCCY7 (*BD* 557871)**
Thy E (Recent Thymic Emigrants)	**CD45RA FITC (*BD* 335039)** **CD31 PE (*BD* 340297)** **CD4 PERCP *(BD* 345770)** **CD8 APC (*BD* 345775)**
XBMEM (Extended B Memory)	**IgD FITC (*CALTAG* H15501)** **CD21 PE (*BD* 555422)** **CD38 PERCPCY5-5 (*BD* 551400)** **CD19 PECY7 (*BD* 341113)** **IgM AF 647 (*STRATECH* 309-605-095)** **CD27 V500 (*BD* 561222)**
Treg (Regulatory T cells)	**CD3BV605 (*BIOLEGEND* 317321) CD4 BV711 (*BIOLEGEND* 317439)** **CD127 FITC (*BIOLEGEND* 351311)** **CD25 PE (*BIOLEGEND* 302605)** **FOXP3 ALEXAFLUOR647 (*BD* 560889)** **CD45RA PECY7 (*BD* 560675)**

Table S2 Clinical Features of Tx patients

Patient	Age at testing (months)	Age at TE (months)	Age at TX (months)	Immune suppression	Clinical features of immunodeficiency	Clinical features of dysregulation	EBV status
1	19	<1	11	ATG			-
2	36	<1	23	ATG	PTLD (5 months post Tx)		+
3	85	70	73	Bas			-
4	81	1	1	Bas	Severe warts		-
5	112	42	46	Bas		Hay fever	+
6	64	21	26	Bas	Early severe CMV		+
7	76	36	42	Bas			+
8	55	3	7	Bas	Early severe CMV		+
9	108	<1	6	Bas	Early severe CMV		+
10	90	7	7	Bas	Recurrent tonsillitis and urinary infections		+
11	112	11	15	Bas	Perioral dermatitis, PTLD (4 months post Tx)	Food allergy	+
12	64	51	56	Bas			+
13	186	168	177	Bas			+
14	147	2	91	Bas			+
15	150	132	143	Bas			-
16	184	118	120	Bas			-
17	199	171	171	Bas			-
18	165	158	158	Bas			-
19	124	15	15	ATG			-
20	180	<1	38	Bas	PTLD (13 years post Tx)	Linear bullous dermatosis, thrombocytopenia	+
21	185	35	35	Bas		Psoriasis	+
22	131	<1	97	ATG	Severe warts		+
23	137	13	14	Bas	Recurrent chest infections	Eczema	-
24	189	<1	112	Bas			-
25*	120	<1	55	Bas			-

TE – Thymic excision
ATG – Initial Immunosuppression with ATG followed by cyclosporine
* This patient had chronic antibody mediated rejection

Table S3 – Ages of patient groups

Age band (years)	Number	Median age (range) at time of testing (months)
		Heart Transplant recipients
1-9	12	78 (19-112)
10-16	13	165 (120-199)
Overall	25	120 (19-199)
		Thymic Excision / no transplant
1-9	7	65 (20-110)
10-16	3	170 (154-196)
Overall	10	101 (20-196)
		Non-thymectomised controls
1-9	18	65 (20-110)
10-16	7	176 (155-201)
Overall	25	97 (13-201)

Figure S1

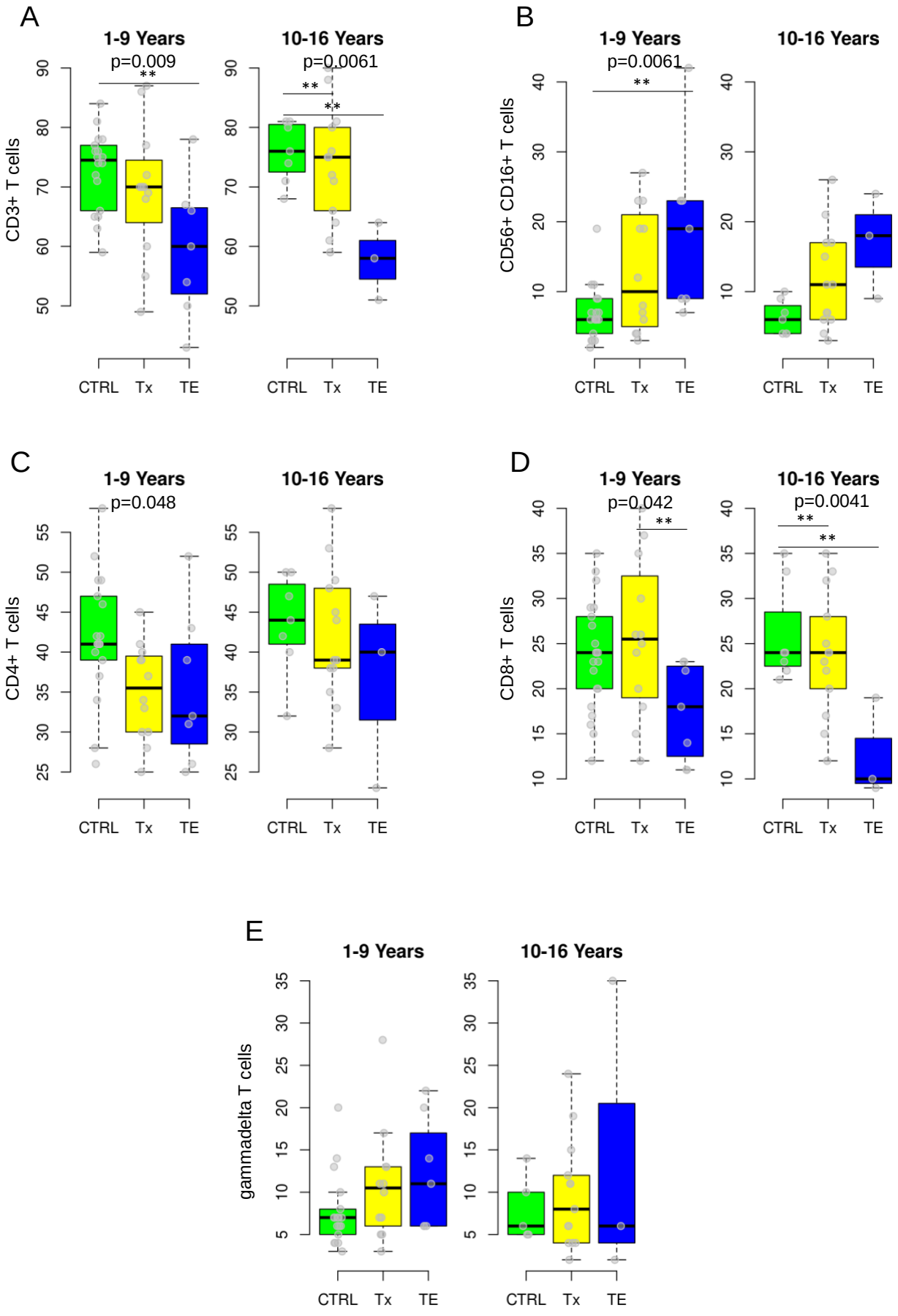

A
1-9 Years
p=0.009
**
10-16 Years
p=0.0061
**
**
CD3+ T cells
50
60
70
80
90
CTRL
Tx
TE
B
1-9 Years
p=0.0061
**
10-16 Years
CD56+ CD16+ T cells
10
20
30
40
C
1-9 Years
p=0.048
10-16 Years
CD4+ T cells
25
30
35
40
45
50
55
D
1-9 Years
p=0.042
**
10-16 Years
p=0.0041
**
**
CD8+ T cells
10
15
20
25
30
35
40
E
1-9 Years
10-16 Years
gammadelta T cells
5
10
15
20
25
30
35

Figure S2

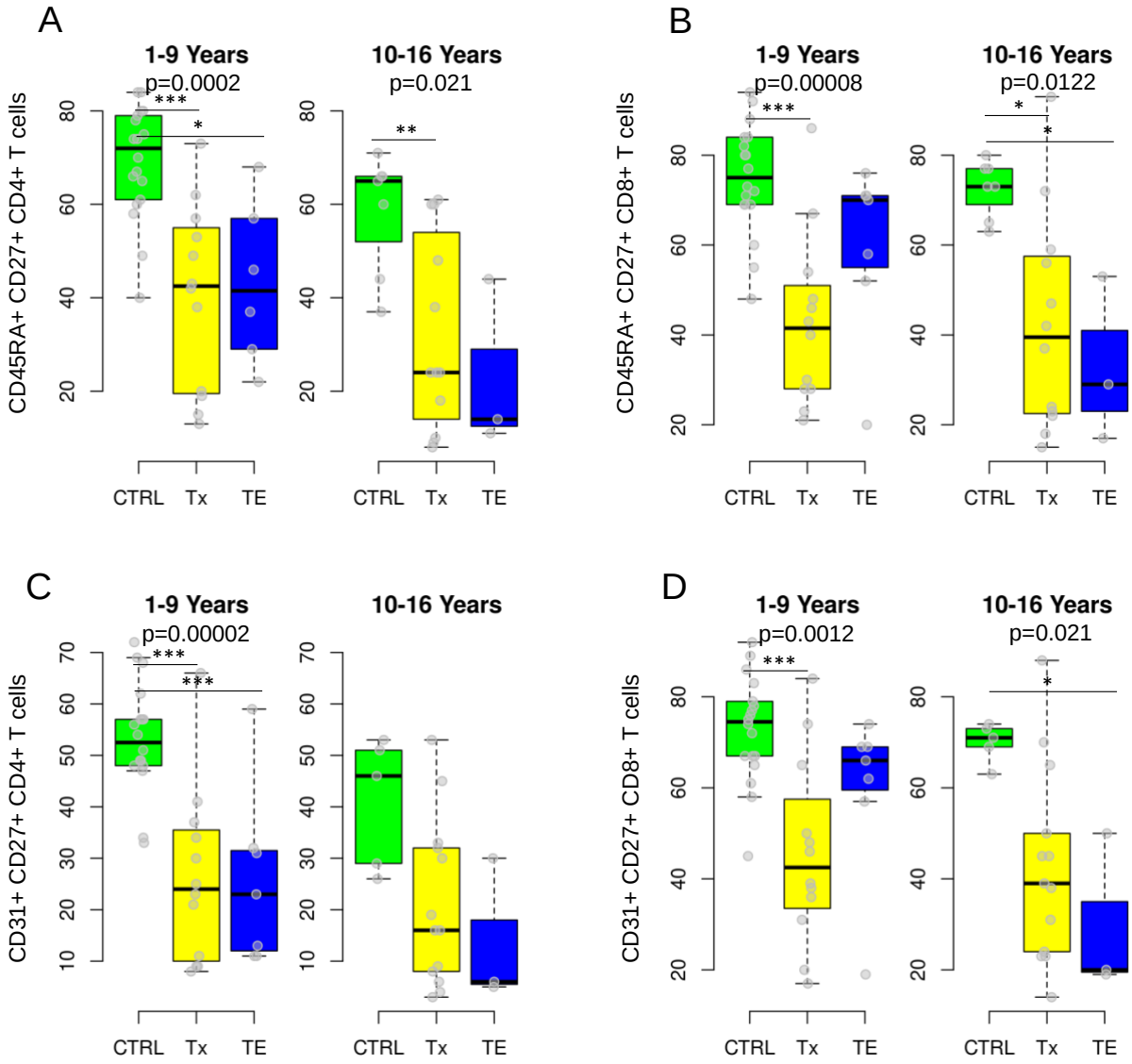
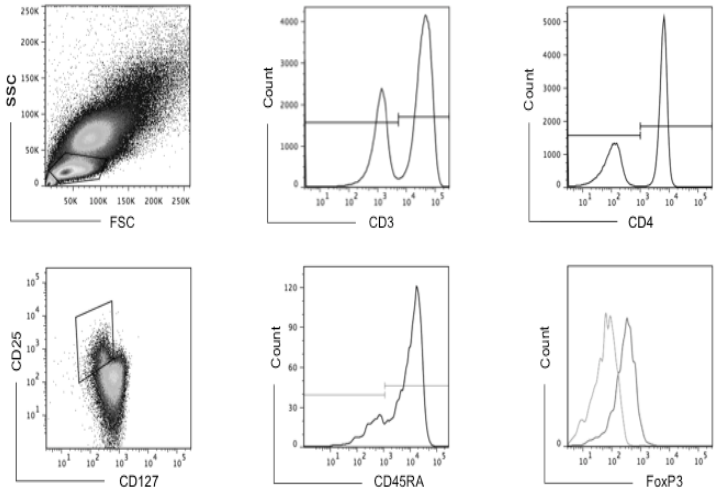

A
1-9 Years
p=0.0002
10-16 Years
p=0.021
CD45RA+ CD27+ CD4+ T cells
B
1-9 Years
p=0.00008
10-16 Years
p=0.0122
CD45RA+ CD27+ CD8+ T cells
C
1-9 Years
p=0.00002
10-16 Years
CD31+ CD27+ CD4+ T cells
D
1-9 Years
p=0.0012
10-16 Years
p=0.021
CD31+ CD27+ CD8+ T cells
CTRL
Tx
TE

Figure S3


SSC
FSC
Count
CD3
Count
CD4
CD25
CD127
Count
CD45RA
Count
FoxP3