

BRIEF COMMUNICATION

Immune dysregulation and Th2 polarization are associated with atopic dermatitis in heart-transplant children: A delicate balance between risk of rejection or atopic symptoms

Jacobo López-Abente¹ | Esther Bernaldo-de-Quirós¹ | Manuela Camino² |
Nuria Gil² | Esther Panadero² | Minia Campos-Domínguez^{1,3} | Elena Seoane-Reula^{1,4} |
Juan M. Gil-Jaurena⁵ | Marjorie Pion¹ | Rafael Correa-Rocha^{1,6} 

¹Laboratory of Immune-regulation, Instituto de Investigación Sanitaria Gregorio Marañón (IISGM), Madrid, Spain

²Pediatric-Cardiology Unit, Hospital General Universitario Gregorio Marañón, Madrid, Spain

³Dermatology Department, Hospital General Universitario Gregorio Marañón, Madrid, Spain

⁴Pediatric Immunology Unit of the Hospital General Universitario Gregorio Marañón, Madrid, Spain

⁵Pediatric Cardiac Surgery Unit of the Hospital General Universitario Gregorio Marañón, Madrid, Spain

⁶Canadian National Transplant Research Program, Canada

Correspondence

Rafael Correa-Rocha
Email: rafael.correa@iisgm.com

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Atopic dermatitis (AD) has a high incidence in heart-transplant children, and the reason why there is more AD after transplantation is still unknown. We conducted a cross-sectional study comparing 11 AD and 11 non-AD age-matched heart-transplant children, to assess which immune alterations are related to AD in these patients. AD patients had been transplanted at a younger age compared to non-AD, indicating that age at transplant may be determinant in the onset of AD. The earlier thymectomy in AD heart-transplant children favored the presence of more differentiated phenotypes in the T cell compartment. We observed a clear reduction in the T-helper 1/T-helper 2 (Th1/Th2) ratio in AD children. This Th2 polarization was related to eosinophilia and high immunoglobulin E levels, but also to an impaired regulatory T cell (Treg) suppression, which could be secondary to an exhaustion of the Treg compartment. Interestingly, AD patients were free of rejection episodes (0/11) in comparison to non-AD children (4/11). We propose that a predominant Th2 phenotype may prevent the emergence of Th1 responses associated with graft rejection. A more differentiated Treg phenotype could also play a role in preventing acute rejection in the first year posttransplant. Our findings provide useful insights and knowledge for the better understanding of atopic disorders in transplanted children.

KEYWORDS

allergy, clinical research/practice, comorbidities, heart transplantation/cardiology, immune regulation, immunobiology, pediatrics, T cell biology, thymus/thymic biology, translational research/science

1 | INTRODUCTION

Heart transplantation is approved for the treatment of end-stage pediatric heart disease. In recent years, improved therapies and clinical practice have led to increased graft survival, and consequently secondary chronic complications have emerged due in part to

long-term immunosuppression.¹ Allergies and atopic diseases, such as atopic dermatitis (AD), are among these chronic complications and constitute important comorbidities in heart-transplant children. AD is a chronic, pruritic inflammatory skin disease of unknown etiology that usually starts in early infancy. Interleukin 4 (IL-4)-secreting CD4⁺ T cells, related to Th2 cells, are considered one of

Abbreviations: AD, atopic dermatitis; CTLA-4, cytotoxic T-Lymphocyte Antigen 4; Foxp3, Forkhead box protein 3; IFN γ , interferon gamma; IgE, immunoglobulin E; IgG₄, immunoglobulin G₄; IL-17, interleukin 17; IL-2, interleukin 2; IL-4, interleukin 4; non-AD, patients without atopic dermatitis; PBMC, peripheral blood mononuclear cells; TemRA, effector memory CD45RA⁺ T cells; Th1, T-helper 1; Th2, T-helper 2; Treg, regulatory T cells.

the principal mediators promoting the immune cascade responsible for inflammation and AD symptoms.²

Regulatory T cells (Treg) are a subset of CD4⁺ T cells with suppressive capacity, which could also play an important role in AD immunopathogenesis. We have shown that high values of IL-4-secreting CD4⁺ T cells in food-allergic children were related to decreased Treg values.^{3,4} However, there is controversy about whether Treg levels are modified in AD patients, and there is little evidence of Treg functional impairment as a mechanism of AD.⁵

The increased incidence of AD in transplanted patients is a highly unexplored area of research. The development of allergies and AD in pediatric heart-transplant patients could have a multifactorial origin because (1) Immunosuppression has proven to be more effective in limiting T-helper 1 (Th1) rather than T-helper 2 (Th2) responses, as reflected by the association between tacrolimus and AD⁶; (2) Use of calcineurin inhibitors impairs Treg-mediated control of Th2 responses leading to allergy or AD⁷; and (3) Early thymectomy in children undergoing cardiac surgery and heart transplantation leads to premature immune aging and a tendency towards a higher incidence of allergies⁸ and therefore it could also increase the AD incidence.

There is some evidence of the role of the Th1/Th2/Treg triangle in AD,^{5,9} but little is known about potential mechanisms underlying AD in children who have undergone transplantation. A better understanding of immune dysregulation in these patients could provide relevant information about AD etiopathogenesis in transplanted children.

2 | MATERIALS AND METHODS

2.1 | Patients and sample processing

Twenty-two consenting heart-transplanted patients were cross-sectionally enrolled at the Pediatric Cardiology Unit of the Hospital "Gregorio Marañón" (HGUGM) and then secondarily grouped by presence of AD. Eleven children were diagnosed with AD after transplant, and 11 heart-transplant children with no record of atopic diseases or allergies were studied as a control group. Diagnosis of AD was made clinically by skin examination and following the Hanifin and Rajka's criteria. Patients affected by AD were treated with topical steroids, which have proven to have a negligible absorption rate. Therefore, a potential effect of this topical treatment on the peripheral immune values was ruled out. All patients were thymectomized (>90% thymic tissue resection). Four patients developed rejection signs in the non-AD group, and samples from these patients were obtained at least 8 weeks after a rejection episode to minimize the effects on immune values of associated inflammation. Rejection was diagnosed by alterations in the electrocardiography and echocardiography monitoring and confirmed by graft biopsy.

The study was conducted after the approval of the HGUGM ethics committee and according to the principles expressed in the Declaration of Helsinki. Written consents from the legal guardians were obtained.

2.2 | Analysis of immune subsets

Fresh peripheral blood samples (<3 mL) were drawn and processed (including staining for flow cytometry) within 2 hours after extraction. Using fresh whole blood, we determined by flow cytometry (Gallios, Beckman Coulter, Villepinte, France) the frequency and absolute counts of CD4⁺, CD8⁺ T cells, and Treg including markers for naive, central memory, effector memory, effector memory CD45RA⁺ T cells (TemRA), and activated cells, as previously described.^{3,10} Percentage and counts of NK, monocytes, dendritic cells, granulocytes, B cells, plasmablasts, and regulatory B cells were also analyzed. Absolute numbers (cells per μ L of blood) of immune subtypes were determined using Flow-Count Fluorospheres (Beckman Coulter) that were added to fresh whole blood samples, providing real/accurate absolute count values.^{3,10}

Percentage of Forkhead box protein 3 (Foxp3) Treg cells (CD3 + CD4 + CD25 + Foxp3⁺) was analyzed in peripheral blood mononuclear cells (PBMC) isolated in a Ficoll-Hypaque gradient using the Anti-Human Foxp3 Kit (eBioscience, San Diego, CA).

Analysis of cytokine-secreting CD4⁺, CD8⁺ T cells, and Tregs was performed in viable PBMC, activated for 5 hours with phorbol myristate acetate (PMA) (50 ng/mL) and Ionomycin (1 μ g/mL). Golgi-stop was added and intracellular staining of IL-2, IL-4, IL-10, IL-17, interferon- γ (IFN- γ), and tumor necrosis factor- α was performed with the Cytofix/Cytoperm Kit (Becton Dickinson, Franklin Lakes, NJ).

2.3 | Analysis of immunoglobulins

Total immunoglobulin E (IgE) and immunoglobulin G4 (IgG₄) were measured in plasma from AD patients using commercial kits (CAP-Phadia, Uppsala, Sweden). Plasma values of IgE and IgG₄ were expressed as kU/L and mg/dL, respectively.

2.4 | Statistical methods

Data distribution was tested for normality by using the Shapiro-Wilk test. Qualitative covariates were compared by the Fisher exact test. The comparison of the different cell-type frequencies among non-AD and AD groups was carried out using weighted median percentages plus interquartile range. The design-based Kruskal-Wallis test was applied as an extension for weighted data of the nonparametric Mann-Whitney test, and an overall 5% significance level was applied. The same nonparametric test was used for the comparison of the different cell types absolute numbers (cells/ μ L) among non-AD and AD groups. Correlations were tested by using the nonparametric Spearman coefficient.

3 | RESULTS

3.1 | Enrolled patients and clinical data

Heart-transplant children with a confirmed clinical diagnosis of AD (n = 11) and a non-AD control group comprising 11 heart-transplant

children were studied. All patients in both groups were thymectomized (with >90% resection of thymic tissue) in the moment of transplant or in previous corrective heart surgeries. AD patients developed atopy after 2.41 ± 0.7 years posttransplant and had total IgE and IgG₄ plasmatic levels at the sampling of 3582 ± 2002 kU/L and 28.4 ± 12.57 mg/dL, respectively. The time elapsed from the transplant and the sampling for the study was comparable in both groups ($P = .645$). The patients in both groups were on similar maintenance immunosuppression therapy, received the same basiliximab induction doses (except 1 non-AD and 2 AD patients who did not receive induction therapy), and tacrolimus blood levels were comparable (Table 1).

There were no significant differences in sex and age between groups (Table 1). However, we found differences in the age at transplantation (non-AD: 3.29 ± 0.8 years; AD: 0.7 ± 0.23 years, $P = .019$). Most of the AD patients (82%) had been transplanted <1 year of age compared to 27% in the control group (Table 1). Age at thymectomy was also lower in the AD group but did not reach statistical significance (non-AD: 2.06 ± 0.76 years; AD: 0.44 ± 0.23 years, $P = .236$).

Even though the time between transplant and this cross-sectional analysis was comparable in both groups (years transplanted, Table 1), there were 4 cases of rejection in the non-AD group compared to the absence of rejection signs in AD patients.

3.2 | Immune status in AD and non-AD heart-transplant children

Percentage and absolute counts (cell/ μ L of blood) of a wide spectrum of immune subsets were analyzed (Table 2). We detected higher frequency ($P = .011$) and absolute counts ($P = .007$) of eosinophils in the AD group, but there were no differences in total B cells, CD4+, CD8+ T cells, monocytes, dendritic cells, basophils, and neutrophils. However, once we analyzed the immune phenotype of these cell subtypes, we detected changes such as higher frequency ($P = .017$) and counts ($P = .034$) of central memory CD4+ T cells, higher frequency of central memory CD8+ T cells ($P = .008$), higher frequency ($P = .038$) and counts ($P = .001$) of plasmablasts, and higher counts of Br1 cells ($P = .017$) in the AD group in comparison to the non-AD group (Table 2). Plasmablasts and Br1 cell values were not correlated with plasma levels of IgE or IgG₄ (data not shown). In summary, even though both patient groups received the same immunosuppression, the AD group presented some clear changes in the immune profile, such as higher levels of memory CD4+ or CD8+ T cells, eosinophils, and plasmablasts.

3.3 | Th1/Th2 imbalance and increased frequency of IL-4-secreting T cells in heart-transplant AD patients

AD has been described as a Th2-driven disease. Thus, we compared the frequencies of different cytokine-secreting CD4+ and CD8+ T cells between the non-AD and AD transplanted children (Figure 1 and Figure S1). The frequency of IL-4-secreting CD4+ T cells (Th2) was significantly higher in AD children when compared

TABLE 1 Clinical parameters of heart-transplant children included in non-AD and AD groups

	Non-AD (n = 11)	AD (n = 11)	P-value
Sex (%)			
Female	3/11 (27%)	0/11 (0%)	.214 ^a
Male	8/11 (73%)	11/11 (100%)	
Age at sampling (y, mean $\pm$ SEM)	7.97 ± 0.61	6.18 ± 1.03	.242 ^b
Age at transplant (y, mean $\pm$ SEM)	3.29 ± 0.8	0.7 ± 0.23	.019 ^b
Age groups at transplant			
≤ 1 y (%)	3/11 (27%)	9/11 (82%)	.03 ^b
>1 y (%)	8/11 (73%)	2/11 (18%)	
Years transplanted (mean $\pm$ SEM)	4.67 ± 0.96	5.48 ± 1.02	.645 ^b
Patients receiving immunosuppressors (%)			
Basiliximab (induction)	10/11 (91%)	9/11 (82%)	1 ^a
Corticoids	2/11 (18%)	1/11 (9%)	1 ^a
Mycophenolate mofetil	8/11 (73%)	7/11 (64%)	1 ^a
Tacrolimus	11/11 (100%)	11/11 (100%)	1 ^a
Tacrolimus level (ng/mL)	6.89 ± 0.52	6.05 ± 0.48	.323 ^b
Azathioprine	0/11 (0%)	1/11 (9%)	1 ^a
Everolimus	1/11 (9%)	0/11 (0%)	1 ^a
Thymectomy (%)			
Pretransplant	6/11 (55%)	7/11 (64%)	1 ^a
At transplant	5/11 (45%)	4/11 (36%)	
Age at thymectomy (y, mean $\pm$ SEM)	2.06 ± 0.76	0.44 ± 0.23	.236 ^b
Signs of rejection (%)	4/11 (36%)	0/11 (0%)	.09 ^a
IgG ₄ (mg/dL)		28.4 ± 12.57	
IgE (kU/L)		3582 ± 2002	

AD, atopic dermatitis; IgE, immunoglobulin E; IgG₄, immunoglobulin G4; non-AD, nonatopic dermatitis; SEM, standard error of the mean.

In bold, P -value < .05.

^aFisher exact test.

^bMann-Whitney test for comparing atopic and nonatopic groups.

to the non-AD group (Figure 1A,D; non-AD: 1.27% [0.55-1.78]; AD: 3.32% [2.08-5.76], $P = .007$). We found a positive correlation between IL-4-secreting CD4+ T cells and eosinophils counts in non-AD and AD groups (Figure S2). A higher frequency of IL-4-secreting cells was also observed in the CD8+ T cell subset (Figure 1B,D, $P = .008$). Interestingly, we detected a significant decrease in the IFN- γ /IL-4-secreting CD4+ T cell ratio (Th1/Th2 ratio) in AD patients (Figure 1E; non-AD: 7.99% [4.1-11.91]; AD:

TABLE 2 Immune parameters in non-AD and AD heart-transplant children

	Percentage, weighted median (IQR)			Cells/ μ L, median (IQR)		
	Non-AD (n = 11)	AD (n = 11)	P-value	Non-AD (n = 11)	AD (n = 11)	P-value
Total lymphocytes						
CD4+ T cells	26.72 (15.23-37.39)	31.82 (26.95-35.13)	.494	624.37 (473.04-693.73)	606.93 (477.69-1061.19)	.438
CD8+ T cells	15.80 (11.43-32.19)	13.58 (9.09-17.73)	.174	465.67 (267.50-651.89)	319.52 (241.03-553.75)	.478
B cells	14.83 (13.37-18.84)	19.36 (15.01-25.10)	.104	325.88 (254.17-409.74)	457.81 (254.58-618.33)	.171
NK	11.20 (9.88-12.74)	11.29 (10.59-20.35)	.174	220.41 (167.76-313.35)	357.53 (260.19-501.39)	.133
CD4+ T cells						
Naive	38.12 (9.16-46.48)	19.14 (16.34-34.47)	.453	235.86 (41.48-333.10)	131.13 (75.07-274.80)	.898
Central memory	46.30 (45.02-48.52)	59.12 (52.36-65.82)	.017	270.23 (246.79-333.30)	370.88 (322.04-587.78)	.034
Effector memory	6.75 (3.39-13.09)	8.29 (7.10-12.77)	.580	45.11 (26.24-82.95)	85.87 (50.71-122.55)	.101
TemRA	0.83 (0.30-1.82)	0.39 (0.14-1.07)	.453	6.45 (2.13-13.11)	4.52 (2.93-8.25)	.606
Activated	1.50 (1.30-2.14)	1.60 (0.61-3.80)	.494	8.49 (7.76-13.48)	16.01 (7.75-17.70)	.332
CD8+ T cells						
Naive	21.13 (9.88-45.41)	27.73 (14.63-41.68)	.922	168.49 (62.73-206.73)	111.32 (71.14-180.50)	.606
Central memory	8.81 (7.09-13.27)	16.04 (10.24-20.86)	.008	63.83 (24.63-69.52)	62.95 (46.98-90.54)	.438
Effector memory	3.24 (1.65-5.02)	5.45 (3.44-6.55)	.249	13.87 (4.21-27.26)	15.28 (7.77-29.71)	.699
TemRA	49.43 (29.63-63.72)	29.32 (15.32-49.54)	.342	177.21 (37.50-400.89)	52.55 (29.56-252.93)	.519
Activated	0.88 (0.48-1.20)	1.87 (0.59-3.33)	.134	4.35 (0.97-7.87)	4.28 (3.51-12.04)	.519
B cells						
Naive	4.70 (1.42-73.80)	4.42 (0.72-10.53)	.494	15.37 (7.16-164.40)	18.76 (8.88-79.93)	.949
Mem. nonswitch	0.89 (0.30-2.10)	0.35 (0.21-0.65)	.278	2.81 (0.72-13.94)	2.49 (0.67-4.38)	.898
Memory switch	10.79 (4.82-17.64)	15.36 (10.12-20.18)	.414	33.40 (14.00-65.70)	49.77 (30.58-98.67)	.171
DN (IgD- CD27-)	66.15 (6.76-80.26)	76.67 (72.58-80.80)	.626	73.05 (29.42-307.67)	283.79 (123.39-479.96)	.076
Plasmablast	1.57 (0.46-2.41)	3.53 (1.38-5.46)	.038	4.54 (3.07-8.65)	10.86 (9.41-25.70)	.001
Br1	0.17 (0.11-0.24)	0.29 (0.15-0.50)	.249	0.71 (0.57-0.87)	1.76 (1.07-2.37)	.017
Breg I	0.51 (0.33-0.54)	0.26 (0.15-0.52)	.626	1.43 (0.62-2.20)	2.26 (1.40-3.21)	.171
Transitional B cells	11.52 (10.08-12.93)	10.39 (6.48-15.86)	.536	43.03 (19.32-55.03)	43.59 (27.63-63.36)	.519
Monocytes	7.81 (5.50-10.00)	8.77 (5.33-11.03)	.871	422.08 (355.87-513.99)	525.16 (408.78-798.09)	.171
Dendritic cells	1.46 (0.97-2.09)	1.52 (0.80-2.36)	.922	78.42 (67.60-121.79)	115.49 (60.56-155.58)	.652
Granulocytes	52.97 (36.53-58.85)	50.85 (39.49-61.88)	.626	2205.79 (1837.12-4092.26)	3681.91 (1973.69-5175.77)	.193
Basophils	0.15 (0.05-0.35)	0.31 (0.10-0.59)	.101	8.05 (2.63-20.36)	27.88 (12.73-36.29)	.101
Activated	13.43 (8.90-32.89)	10.02 (8.06-12.46)	.494	2.66 (0.48-3.41)	2.76 (2.46-5.40)	.270
Eosinophils	1.68 (0.73-6.27)	5.66 (3.50-13.13)	.011	75.84 (65.19-245.14)	470.48 (238.43-880.38)	.007
Neutrophils	48.77 (30.50-52.47)	42.24 (28.20-48.06)	.580	2132.99 (1557.24-3686.08)	2603.55 (1684.82-4220.31)	.519

Percentages and absolute counts (cells per μ L of total blood) for immune populations are shown. The percentage of each subset is calculated regarding the total of the corresponding population (total lymphocytes, CD4+ T cells, CD8+ T cells, B cells). Percentages of monocytes, dendritic cells, granulocytes, basophils, eosinophils, and neutrophils are calculated over total leukocytes (CD45+). Values are given as weighted median (IQR) and median (IQR) for percentages and absolute counts, respectively. P-values from nonparametric Wilcoxon-Mann-Whitney test comparing non-AD and AD groups. In bold, P-value <.05. AD, atopic dermatitis; Breg, regulatory B cells; IQR, interquartile range; non-AD, nonatopic dermatitis; TemRA, effector memory CD45RA+ T cells.

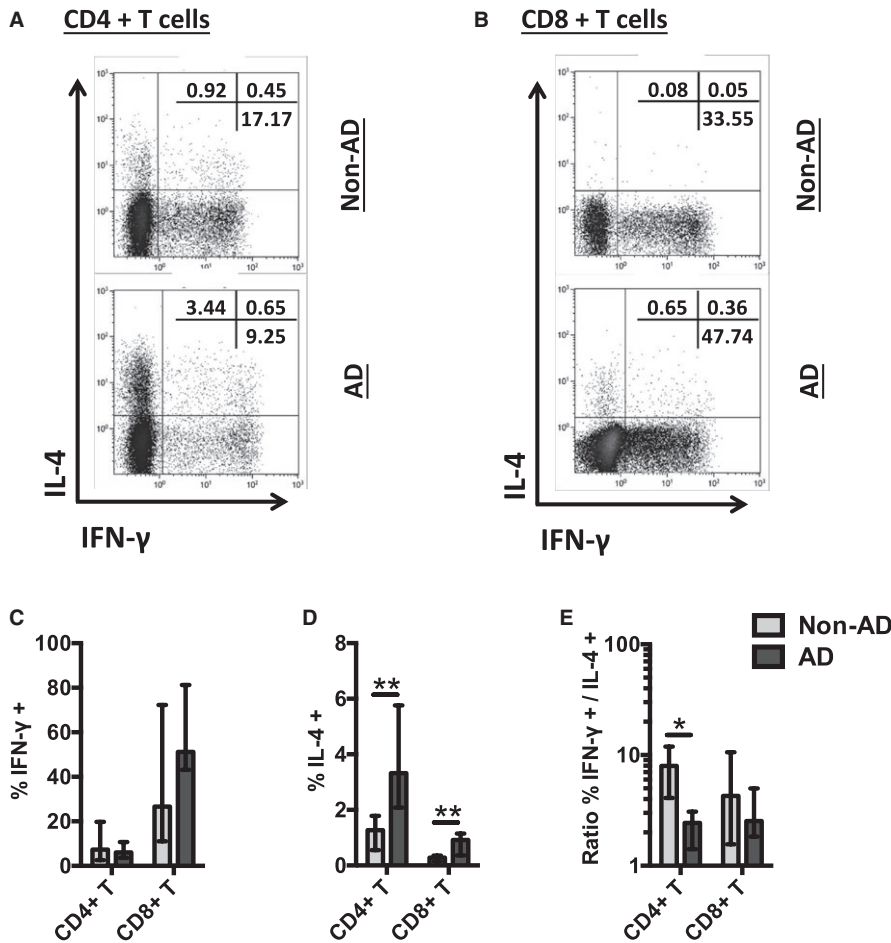


FIGURE 1 High frequency of IL-4-secreting T cells and Th1/Th2 imbalance in the AD heart-transplant children. Dot plots of IL-4- and IFN- γ -secreting CD4+ (A) and CD8+ T cells (B) are shown as a representative example. Numbers represent the percentage of cell populations in each quadrant. Weighted median (IQR) frequency of IFN- γ - (C) and IL-4- (D) secreting CD4+ and CD8+ T cells. IFN- γ /IL-4-secreting CD4+ and CD8+ T cell ratio (E). Gray bars: non-AD group (n = 11). Black bars: AD group (n = 11). For TNF- α conditions n = 10 in each group is represented. * $P < .05$, ** $P < .01$ in the nonparametric Wilcoxon-Mann-Whitney test comparing non-AD and AD values. AD, atopic dermatitis; IL-4, interleukin 4; IFN- γ , interferon- γ ; IQR, interquartile range; Th1/Th2, T-helper 1/T-helper 2; TNF- α , tumor necrosis factor- α

2.43 [1.41-3.08], $P = .020$). Additionally, we observed higher frequency of IL-2-secreting CD4+ T cells in AD patients (Figure S1, $P = .014$). We found no differences in the rest of the analyzed cytokines in both CD4+ and CD8+ T cells (Figure 1 and Figure S1).

3.4 | Treg values and phenotype in heart-transplant AD patients

The frequency and absolute counts of Tregs were similar in both groups (Table 3, $P = .249$ and $P = .243$, respectively). AD patients had higher absolute counts of central memory ($P = .023$), effector memory ($P = .007$), and TemRA Treg cells ($P = .018$) in comparison to the non-AD group. We also analyzed Treg cells in regard to the expression of Foxp3 and other markers related to their suppressive capacity (CD39, cytotoxic T-lymphocyte antigen 4 [CTLA-4]). Whole blood Treg (CD3 + CD4 + CD25 + CD127⁻) and Foxp3 + Treg (CD3 + CD4 + CD25 + Foxp3⁺) from isolated PBMC were highly correlated and comparable (data not shown) as already described. There were no differences in the frequency of CD4 + CD25 + Foxp3⁺ cells (Figure 2A, $P = .580$) nor in Foxp3 expression on a per cell basis (Foxp3 MFI, Figure 2B, $P = .237$). However, we detected an increase in the frequency of CTLA-4⁺ cells in the Foxp3⁺ Treg subset of the AD group ($P = .038$; Figure 2C).

The frequency of IL-10⁻, IL-4⁻, and IL-17-secreting Foxp3⁺ Treg cells was analyzed in order to define 2 different aspects: the suppressive potential of Tregs through IL-10 secretion, and a potential switch of Treg cells to effector non-Treg phenotypes through IL-4 or IL-17 secretion. However, we could not detect any differences between either of the groups at the Treg cytokine-secretion profile (Figure 2D).

3.5 | Correlation between Tregs and the frequency of effector immune subsets is lost in transplanted AD patients

Non-AD patients presented a clear inverse correlation between the frequency of IL-4-secreting CD4+ T cells and Treg counts ($P = .001$; Figure 3). Similar negative correlations were observed in non-AD patients when comparing Treg counts and the frequency of effector memory, IFN- γ and IL-17-secreting CD4+ T cells (Table S1). However, in AD transplanted children, all the aforementioned associations were lost (Figure 3, Table S1). In summary, strong correlations between Treg and effector or pro-inflammatory phenotypes, which are indicative of the suppressive control exerted by Treg over T cells, were lost in AD heart-transplant patients.

TABLE 3 Values of Treg subsets in non-AD and AD heart-transplant children

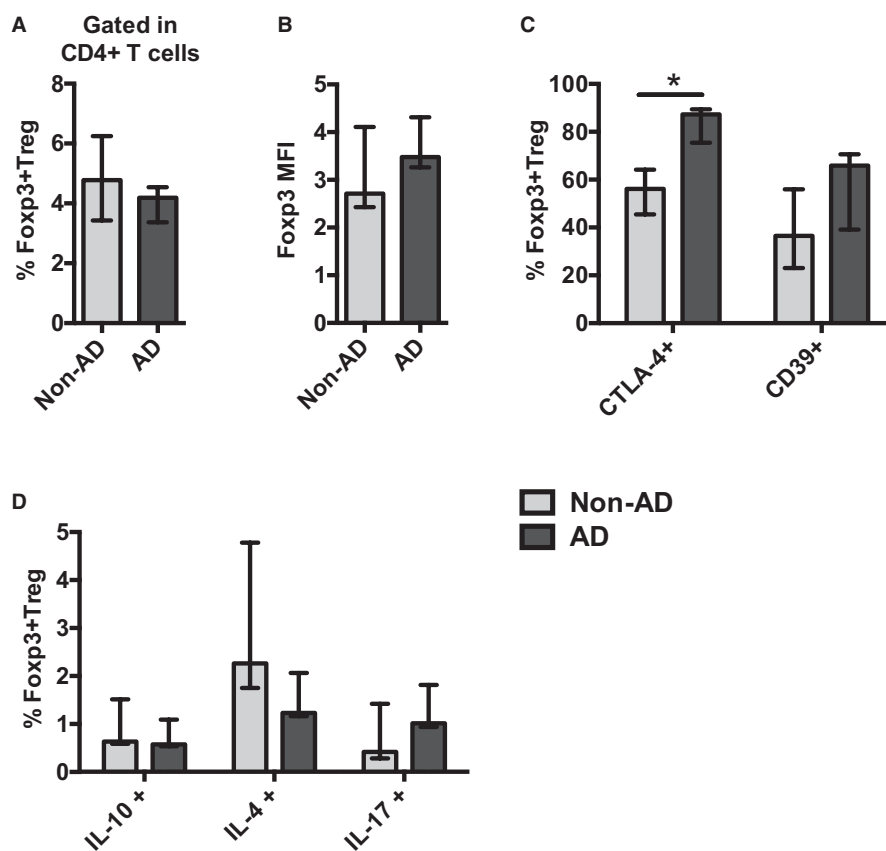
	Percentage, weighted median (IQR)			Cells/ μ L, median (IQR)		
	Non-AD (n = 11)	AD (n = 11)	P-value	Non-AD (n = 11)	AD (n = 11)	P-value
Treg ^a	3.50 (1.86-4.52)	4.12 (3.65-4.25)	.249	23.82 (10.26-30.53)	26.93 (21.05-31.82)	.243
Naive ^b	38.61 (17.51-58.54)	16.81 (13.60-20.11)	.820	4.39 (0.55-13.43)	5.11 (2.52-11.57)	.847
Central memory ^b	48.19 (26.18-71.46)	66.65 (55.79-72.99)	.820	9.43 (8.18-16.00)	15.97 (14.28-21.14)	.023
Effector memory ^b	4.08 (1.99-5.24)	7.43 (5.95-12.30)	.453	1.02 (0.76-1.26)	2.03 (1.48-3.42)	.007
TemRA ^b	0.07 (0.00-0.12)	0.14 (0.07-0.21)	.172	0.02 (0.00-0.03)	0.05 (0.03-0.08)	.018
Activated ^b	9.00 (6.11-12.66)	8.80 (3.95-19.60)	.770	1.94 (1.64-2.97)	2.73 (1.76-3.53)	.519

Percentages and absolute counts (cells per μ L of total blood) of CD4 + CD25 + CD127⁻ cells (Treg) and Treg subsets are shown. P-values from nonparametric Wilcoxon-Mann-Whitney test comparing non-AD and AD groups. In bold, P-value <.05. AD, atopic dermatitis; IQR, interquartile range; non-AD, nonatopic dermatitis; TemRA, effector memory CD45RA⁺ T cells; Treg, regulatory T cells⁺.

^aGated in total CD4⁺ T cells.

^bGated in Treg. Values are given as weighted median (interquartile range) and median (interquartile range) for percentages and absolute counts, respectively.

FIGURE 2 Foxp3⁺ Treg functional phenotyping in non-AD and AD heart-transplant children. Frequency of CD4⁺ CD25⁺ Foxp3⁺ T cells (Foxp3⁺ Treg), gated in CD4⁺ T cells (A) and Foxp3⁺ median fluorescence intensity (MFI) in Foxp3⁺ Treg (B). Frequency of CTLA-4⁺ and CD39⁺ gated in Foxp3⁺ Treg in non-AD (gray bars, n = 11) and AD (black bars, n = 11) group (C). IL-10⁻, IL-4⁻, IL-17-secreting Foxp3⁺ Treg in non-AD (n = 5) and AD (n = 6) group (D). Values are given as median (interquartile range). *P < .05 in nonparametric Mann-Whitney test comparing non-AD and AD values. AD, atopic dermatitis; CTLA-4, cytotoxic T-lymphocyte antigen 4; Foxp3⁺, Forkhead box protein 3; IL-4, interleukin 4; IL-10, interleukin 10; IL-17, interleukin 17; MFI, median fluorescence intensity; non-AD, nonatopic dermatitis; Treg, regulatory T cells



4 | DISCUSSION

There have been many attempts to understand the association between atopic diseases and solid organ transplantation, and it is likely to be multifactorial. In the context of pediatric heart transplantation, an additional factor is the thymectomy, which has a profound impact on pediatric patients' immune systems. A higher incidence of allergies has been shown in children who have undergone thymectomy before the age of 6 months but not in older age groups.^{8,11} Early thymectomy also results in reduced

TCR diversity,¹² which has been associated with AD.¹³ The impaired thymic function has also been related to a higher incidence of autoimmune and atopic diseases, as shown in children with thymic hypoplasia secondary to partial Di-George syndrome,^{14,15} and even in pediatric heart transplant patients.^{1,16} In agreement with all these findings, our results indicate that age at time of heart transplantation and subsequent thymectomy seems to be a crucial factor for AD development, as patients who developed AD had been transplanted at younger ages than non-AD patients.

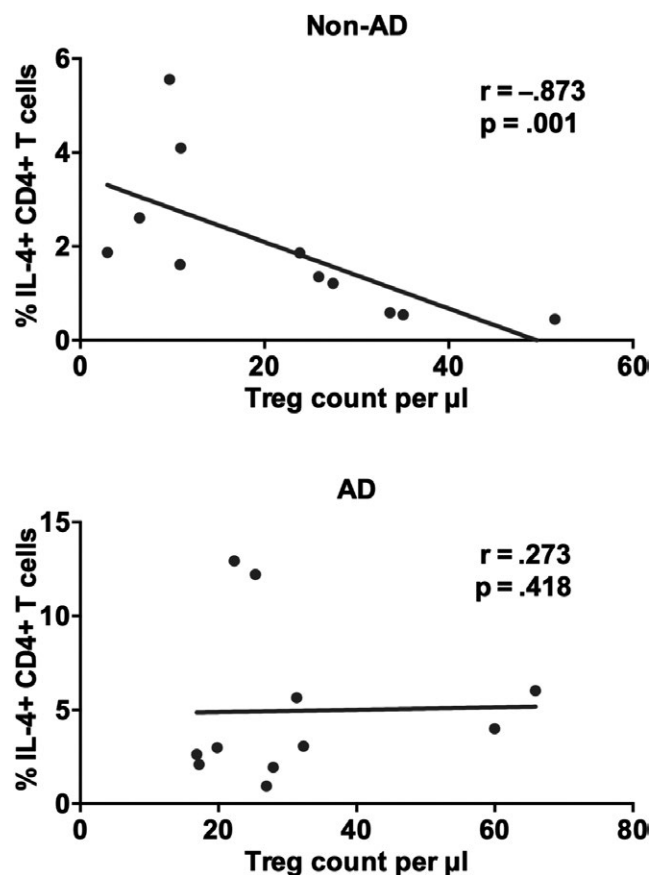


FIGURE 3 Association loss between Treg and IL-4-secreting CD4+ T cells in AD patients. Correlations between CD4+ CD25+ CD127- (Treg) count per μ L and the frequency of IL-4-secreting CD4+ T cells in non-AD (upper graph) and AD groups (lower graph). Correlations were tested by using the Spearman coefficient (r : Spearman's correlation coefficient). $P < .05$ was considered significant. AD, atopic dermatitis; IL-4, interleukin 4; non-AD, nonatopic dermatitis; Treg, regulatory T cells

Early thymectomy leads to an increase in memory T cells as a result of a compensatory homeostatic proliferation in response to the lack of naïve T cell renewal.^{8,11} This phenomenon is reflected in increased values of the more differentiated phenotypes of CD4+, CD8+ T cell and Treg populations observed in our cohort of AD patients, which would have a clear impact on the immune homeostasis.

In contrast to the results shown in the literature, there was no association between an earlier thymectomy in AD patients and lower Treg numbers.⁸ Because high Treg values have been associated with AD,⁵ a potential explanation is that AD-related Treg expansion may compensate the negative effect of early thymectomy over Treg levels. In fact, the higher frequency of IL-2-secreting cells observed in AD patients could be responsible for a higher activation, proliferation, and differentiation of Tregs, due to the dependence of Tregs on IL-2.¹⁷ As a consequence, Treg cells presented a more differentiated phenotype in the AD group, and central and effector memory Tregs comprised up to 75% of the total Treg population compared to the 55% in non-AD patients. However, this increased differentiation could also lead to Treg exhaustion,¹⁸ compromising their role in preserving immune homeostasis.

Indeed, we observed a clear switch towards Th2 responses in AD heart-transplant patients. Others have shown an insufficient control of allergen-specific Th2 responses in transplanted patients related to a deficit in the Treg-cell compartment under systemic immunosuppression.¹⁹ We observed a lack of correlation between Treg and IL-4-secreting CD4+ T cells in AD patients, which in contrast was preserved in non-AD transplanted children. This fact could be indicative of a failure to control Th2 responses by Tregs, leading to higher IL-4-secreting CD4+ T cell levels (Th2 polarization), a Th1/Th2 imbalance, and ongoing AD.

Th2 polarization could be the origin of other immune alterations observed in AD patients. IL-4 enhances IgE production, while IFN- γ , secreted by Th1 cells, is suggested to inhibit IgE synthesis.²⁰ Consequently, total serum IgE is elevated in 80%-90% of AD patients and their levels correlate with disease severity.²¹ Additionally, Th2 activation leads to eosinophil proliferation and selective recruitment to inflamed skin, where they mediate the release of granules and tissue damage.²² This fact may explain the positive correlation shown between IL-4-secreting cells and increased values of eosinophils in our AD patients.

Regarding the B cell population, it has been shown in adults that AD is accompanied by a systemic expansion of transitional or memory B cells and plasmablasts,²³ a proliferating fraction of antibody-secreting B cells. We detected higher plasmablast levels, which could be related to the fact that AD patients had plasma IgE levels >300 times higher than the reference values reported in healthy children.²⁴

Despite the relatively low number of patients enrolled in this study, it was an interesting observation that none of the AD patients experienced rejection episodes. This fact could have 2 possible interpretations; first, AD patients were younger at transplantation, and an association between younger age at transplantation and lower risk of rejection has been described.²⁵ Second, Th2 dominant phenotypes in AD patients could be associated with a lower risk of rejection, which agrees with other studies showing that Th2 responses are predominant in nonrejecting patients. For example, it has been found that a predominant Th2 phenotype is accompanied by decreased Th1 responses, which are associated with decreased risk of rejection in pediatric liver transplantation.²⁶ Additionally, eosinophils have immunomodulatory activity through the secretion of indoleamine 2,3 dioxygenase, leading to kynurenine production, which has anti-Th1 activity.²⁷ Therefore, high eosinophil counts, as observed in our AD cohort, has been associated with an absence of rejection in heart-transplant pediatric recipients,²⁸ strengthening the association between eosinophilia, AD development, and freedom from rejection.

Another interesting point discovered by our work was the presence of higher counts of Tregs with an effector phenotype (effector memory and TemRA) in AD patients. It has already been described that the presence of Tregs was associated with the prevention of rejection in heart transplantation.²⁹ Therefore, even if Treg cells appear to be exhausted and unable to control Th2 expansion at the time of this study (>4 years posttransplant), it is possible that

a higher presence of effector Treg in the first years after transplantation was able to suppress Th1 responses and thus, prevent acute rejection before the exhaustion of the Treg population. Moreover, there are cases where Tregs may have selectively impaired control over Th2 responses, but not Th1 responses.³⁰

Summing up, the younger age at transplantation and subsequent thymectomy, the dominance of a Th2 response, and an apparent inability of Treg cells to prevent a Th1/Th2 imbalance could constitute the main mechanisms responsible for the onset of AD in heart-transplant children that has been reported by several authors.^{1,16} The Th2 polarization may be related to eosinophilia, increased plasmablast counts, and high IgE titers in AD patients, all of which are probably contributing to the ongoing AD disease. However, the same Th2 polarization could be beneficial in terms of reducing the risk of rejection. Graft rejection is more related to Th1 responses, and a switch towards a Th2 phenotype with the potential protective role of increased effector Tregs in the first couple of years posttransplant could contribute to prevent acute rejection.

4.1 | Limitations of the study

Due to the low number of pediatric heart transplants performed worldwide, the number of available patients to perform this kind of study is very limited. The low sample size and heterogeneity of the cohort groups makes it challenging to provide robust, reliable conclusions.

Confounding factors such as the difference in age at transplant and age at thymectomy were different comparing our patient groups, although below significance level, likely due to the small sample size. However, our immunological data offer a potential model for age and thymectomy-dependent changes in the immune system, resulting in higher likelihood to develop AD.

Further research in this area will provide new avenues to identify potential predictive or diagnostic markers of interest in the clinical follow-up of transplanted patients, and even novel immunological mechanisms associated with graft tolerance.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*. Data available on request due to privacy/ethical restrictions.

ORCID

Rafael Correa-Rocha  <https://orcid.org/0000-0003-3456-9986>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Figure S1

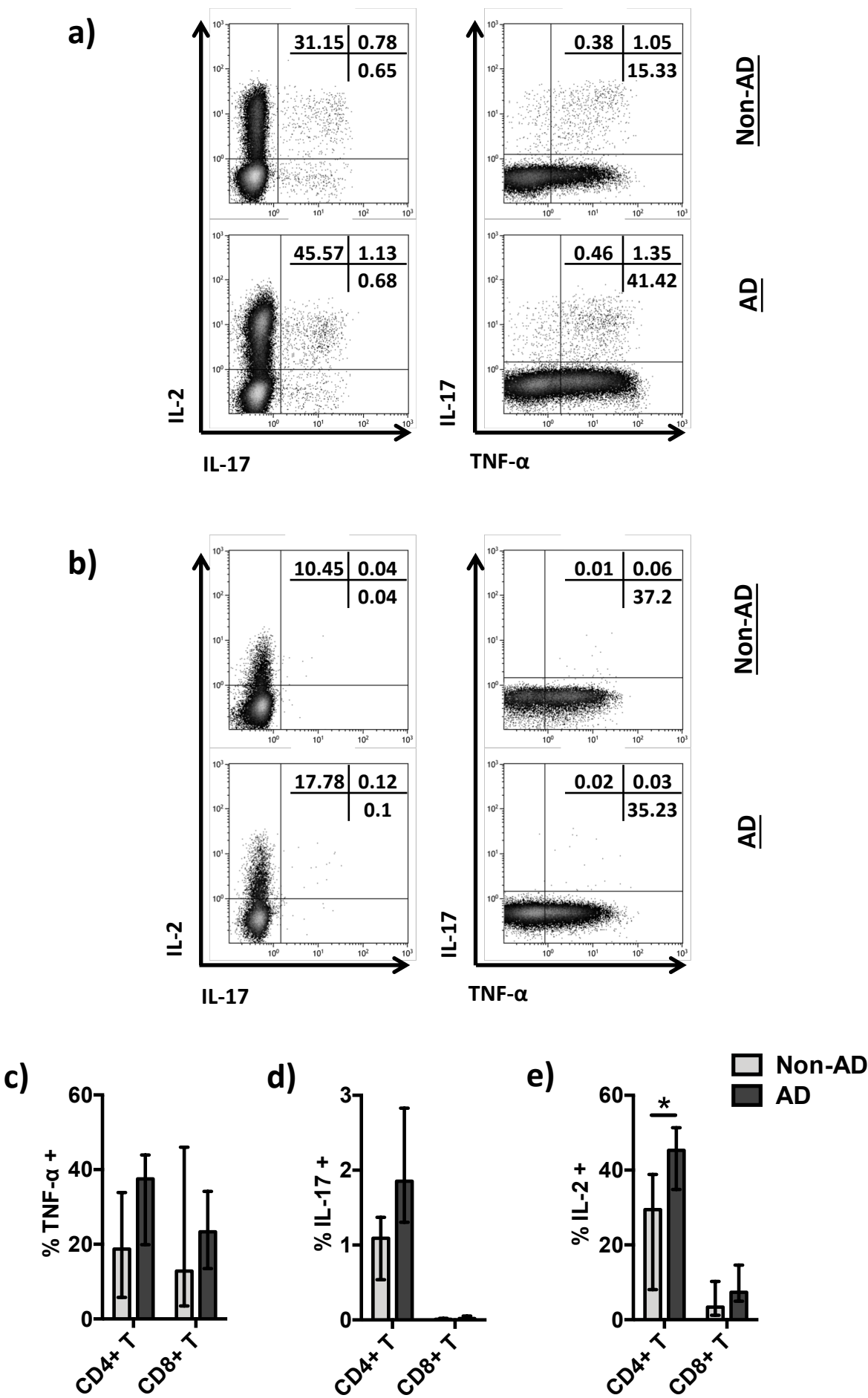


Figure S2

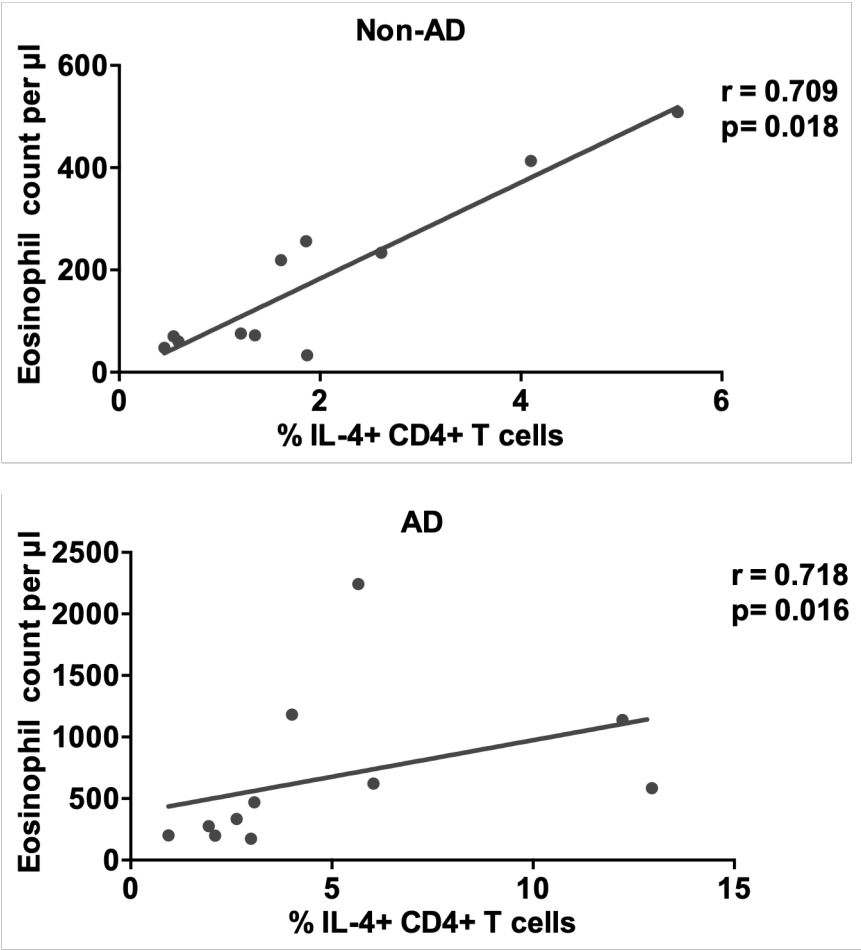


Table S1. Correlations between Treg and effector, cytokine-producing CD4+ and CD8+ T cells. Correlations between CD4+CD25+CD127– and effector memory (EM), IFN- γ -, TNF- α -, IL-2- and IL-17-cytokine producing CD4+ and CD8+ T cells in non-AD (non-atopic dermatitis) and AD (atopic dermatitis) group. Correlations were tested by using the Spearman's correlation coefficient. $p < 0.05$ was considered significant.

Condition 1	Condition 2	Non-AD		AD	
		Correlation	p-value	Correlation	p-value
Treg cell/ μ l	% EM CD4+ T cells	-0.809	0.004	-0.436	0.183
	% IFN- γ + CD4+ T cells	-0.736	0.013	-0.37	0.296
	% TNF- α + CD4+ T cells	-0.576	0.088	0.091	0.811
	% IL-2+ CD4+ T cells	-0.373	0.261	0.045	0.903
	% IL-17+ CD4+ T cells	-0.791	0.006	0.336	0.313
	% EM CD8+ T cells	-0.645	0.037	-0.282	0.400
	% IFN- γ + CD8+ T cells	-0.255	0.451	0.018	0.973
	% TNF- α + CD8+ T cells	-0.103	0.785	0.176	0.632
	% IL-2+ CD8+ T cells	0.036	0.924	0.218	0.521
	% IL-4+ CD8+ T cells	-0.327	0.327	-0.105	0.759
	% IL-17+ CD8+ T cells	-0.258	0.443	-0.207	0.541

SUPPLEMENTAL FIGURES

Supplemental Figure S1. Frequency of cytokine-secreting T cells in the AD (atopic dermatitis) heart-transplant children. Dot plots of IL-2- IL-17- and TNF- α -secreting CD4+ (a) and CD8+ T cells (b) are shown as a representative example. Numbers represent the percentage of cell populations in each quadrant. Percentage of TNF- α - (c), IL-17- (d) and IL-2- (e), secreting CD4+ and CD8+ T cells. Grey bars: Non-AD group (n= 11). Black bars: AD group (n= 11). For TNF- α + conditions n= 10 is represented. Values are given as weighted median (IQR). *p<0.05 in the non-parametric Wilcoxon-Mann-Whitney test comparing non-AD and AD values.

Supplemental Figure S2. Correlation between IL-4-secreting CD4+ T cells and eosinophil count (cell per μ l of blood). Correlations between the frequency of IL-4-secreting CD4+ T cells and eosinophil counts per μ l in non-AD (non-atopic dermatitis, upper graph) and AD groups (atopic dermatitis, lower graph). Correlations were tested by using the Spearman coefficient (r: Spearman's correlation coefficient). p<0.05 was considered significant.