

Changes in thymic regulatory T-cell maturation from birth to puberty: Differences in atopic children

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Background: Characterization of regulatory immune pathways is a research priority for both the pathogenesis of allergic disease and potential therapeutic strategies.

Objective: The thymus is a rich source of regulatory T (Treg) cells, which offers a novel opportunity to document the maturation of these pathways beyond limited studies on small volumes of peripheral blood available from young children.

Methods: Thymus tissue was collected from children undergoing cardiac surgery (age, 1 week to 14 years), and skin prick testing was performed from 12 months of age. The ontogeny of Treg cell maturation and function was examined in atopic (n = 20) and nonatopic (n = 20) children by assessing their phenotype, enumeration, proliferation, and suppressive ability.

Results: Age-related changes in the thymic cytokine milieu paralleled the changes seen in peripheral immune function. Specifically, the thymic microenvironment is similarly T_H2 skewed during the early postnatal period, and this undergoes age-related suppression as the T_H1 (IFN- γ) response increased. We detected CD4⁺CD25⁺CD127^{low/-} forkhead box protein 3 (FOXP3)-positive Treg cells in the neonatal thymus. These cells suppressed the proliferative response to allogeneic stimulation of CD4⁺CD25⁻ T cells dose dependently. In nonatopic children Treg cell turnover and suppressive function increased with age and paralleled the increase in global thymic FOXP3 mRNA expression, whereas in atopic children Treg cell maturation was significantly delayed compared with that seen in age-matched nonatopic children.

Conclusion: These data suggest that the developmental changes in the thymus parallel the recognized changes in peripheral blood responses. There is also a developmental delay in the function of thymic regulatory cells in atopic compared with

nonatopic children. These differences are fundamental to understanding early events that lead to immune dysregulation and might predispose to allergic disease. (*J Allergy Clin Immunol* 2011;■■■■:■■■-■■■.)

Key words: Regulatory T cells, thymus, immune maturation, children, allergy, sensitization, atopy

Immune-regulatory mechanisms are believed to play a pivotal role in suppressing excessive, inappropriate, or misdirected immune responses that can lead to both allergic and autoimmune diseases.¹ The dramatic recent increase in most broad-ranging immune disorders indicates the high susceptibility of regulatory pathways to environmental changes.² Although developmental differences in regulatory pathways are deemed of likely importance in allergy pathogenesis and susceptibility to environmental change,³ their ontogeny in the early postnatal period is not documented.

Immune homeostasis is achieved through various regulatory mechanisms, including CD4⁺CD25⁺ regulatory T (Treg) cells, as reviewed by Piccirillo.⁴ Treg cells play a pivotal role in maintaining immunologic unresponsiveness to innocuous self-proteins and environmental proteins and in suppressing excessive immune responses deleterious to the host. Because isolating a sufficient number of Treg cells from small volumes of peripheral blood in children to assess regulatory function is a challenge, we turned to the use of freshly isolated thymic tissue as a rich source of Treg cells.

Naturally occurring CD4⁺ Treg cells are derived centrally in the thymus and constitutively express CD25 (IL-2 receptor α chain), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and surface TNF receptor 2.⁵ CD25⁺ Treg cells exert suppressive effects through direct cell contact. The gene encoding forkhead box protein 3 (FOXP3) is a critical regulator of CD4⁺CD25⁺ naturally occurring CD4⁺ Treg cells. FOXP3⁺ natural Treg cells (CD4⁺CD25⁺FOXP3⁺) are functionally mature and antigen primed in the thymus.⁶

Whether allergic diseases are associated with underlying deficiencies in allergen-specific Treg cell numbers, function, or both remains contentious.^{7,8} In part, this might be due to difficulties in accurately identifying CD4⁺ Treg cells by using conventional cell markers. CD4⁺ T cells typically express CD25, as do activated effector T cells. Similarly, effector T cells might transiently express FOXP3.⁹ More recently, CD127 (the IL-7 receptor α chain) negative expression can discriminate Treg cells from activator T cells.¹⁰ We recently used CD4⁺CD25⁺CD127^{low/-} markers to determine perinatal differences in Treg cell function in ensuing allergic disease.¹¹

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Abbreviations used

CTLA-4: Cytotoxic T lymphocyte-associated antigen 4

FOXP3: Forkhead box protein 3

SEB: Staphylococcal enterotoxin B

SPT: Skin prick test

Treg: Regulatory T

TSLP: Thymic stromal lymphopoietin

TSLPR: Thymic stromal lymphopoietin receptor

We characterized changes in the phenotype and function of thymic Treg cells from healthy nonatopic children and compared these with changes seen in age-matched atopic children. Our novel findings support our hypothesis that atopic compared with nonatopic children show a relative delay in the maturation of Treg cell function. This study addresses the need to characterize the development of regulatory pathways in early life, when susceptibility to environmental effects might be crucial.

METHODS**Subjects**

Children (age, 1 week to 14 years) undergoing cardiopulmonary bypass surgery were recruited from Princess Margaret Hospital in Perth, Australia. The thymus is routinely removed to provide surgical access and is usually discarded. We included only children who are otherwise well and not receiving immunomodulatory medication. Children who had significant immune disorders, including those associated with their cardiac defect (eg, DiGeorge anomaly) or another condition likely to be associated with impaired immune function, were excluded. All patients were free of viral/bacterial infections during the month before surgery. Blood (5–10 mL) was collected during surgery after achievement of anesthesia with 100% success (indwelling catheters or central lines are in place). Informed consent was obtained from all parents. The study was approved by the Princess Margaret Hospital Ethics Committee and was conducted according to the Helsinki protocol and National Health and Medical Research Council guidelines.

Clinical follow-up and allergy testing

In a cross-sectional study design we aimed to recruit 20 atopic and 20 age-matched nonatopic children, with equal numbers in each of 4 age groups (<6 months, 6–12 months, 1–5 years, and >5 years). The main objective of the study was to compare children with an established propensity for allergen-specific IgE production (reflecting underlying proallergic T_H2 responses) with nonallergic children. Thus the main criterion for defining atopy was the presence of sensitization to 1 or more allergens on a skin prick test (SPT).

Additional information on symptomatic disease was also collected from questionnaires and clinical history at the follow-up visit. Because the child's allergic phenotype was unknown at the time of surgery, all families were invited to attend separate clinical visits to ascertain their allergic status and complete interview questionnaires. The questionnaires documented any clinical evidence of allergic disease, information on general health, indications for surgery, medications, and environmental exposures.

Allergy assessments were performed from 12 months of age, and the younger children (ie, <6 months of age at surgery) were invited to return for clinical follow-up after their first birthday. The main allergic outcomes were eczema, IgE-mediated food allergy, and sensitization. For clinical definitions, see the Methods section in this article's Online Repository at www.jacionline.org.

Sensitization

SPTs were performed by using a standardized technique with common allergen extracts (egg, milk, peanut, house dust mite, cat, grass, and mold; Hollister-Stier Laboratories, Spokane, Wash), as well as histamine (positive control) and glycerin (negative control). A wheal diameter of 3 mm or greater was considered positive.

Tissue preparation

Surgically resected thymus (approximately 1 cm^3) was homogenized into a single-cell suspension and cryopreserved in FCS and 15% dimethyl sulfoxide for future batch analysis by using established techniques.¹² A separate piece was processed for histology and immunocytochemistry (see below). Cryopreserved thymocytes were used for cell culture, flow cytometry, and mRNA extraction, as previously described.¹³ Routinely, thymus tissue was processed within 60 minutes of excision.

Whole thymocyte cultures

Thymocytes ($1 \times 10^6/\text{mL}$) were cultured in 96-well round-bottom culture plates (NUNC, Roskilde, Denmark) in AIM-V serum-free medium (Gibco Life Technologies, Mulgrave, Australia) supplemented with 2-mercaptoethanol ($4 \times 10^{-5}\text{ mol/L}$; Sigma, Castle Hill, Australia) to assess normal maturation of T-cell responses with age and the effect of allergic sensitization. Cultures were performed in a final volume of 200 μL at 37°C with 5% CO_2 either alone (control) or stimulated with PHA mitogen (1 $\mu\text{g/mL}$; HA16, Murex, Biotech Ltd, Dartford, United Kingdom) or staphylococcal endotoxin B (SEB; 200 ng/mL; Sigma-Aldrich, St Louis, Mo) for 48 hours. Supernatants were collected and stored at -20°C for cytokine measurements. Cell pellets were resuspended in 100 μL of RNeasy Lysis Buffer (Qiagen, Crawley, Australia) and stored at -20°C for RNA extraction and RT-PCR.

Flow cytometry

CD127 (the IL-7 receptor α chain) better discriminates Treg cells (with significantly lower CD127 expression) from conventional activator T cells.¹⁰ We recently used this combination of markers (ie, $\text{CD4}^+\text{CD25}^+\text{CD127}^{\text{low/-}}$) to examine perinatal differences in Treg cell function in association with subsequent allergic disease.¹¹ The proportion of Treg cells in the thymus (as a percentage of total thymocytes and a percentage of total CD4^+ T cells) at different ages was examined by labeling thymocytes with mAbs directed against CD4-AF488 (1:10), CD25-PECy5 (1:5), and CD127-phycoerythrin (1:10; BD Biosciences, San Jose, Calif) for 30 minutes on ice in the dark and then permeabilized (Cytofix/Cytoperm buffer; BD Pharmingen, San Jose, Calif) for 30 minutes before staining with FOXP3-allophycocyanin (1:25; BD, Franklin Lakes, NJ). In some samples Treg cells were stained with Ki67-PECy7 (1:100) as a measure of cell turnover. Cells were washed in RPMI 2% FCS (Australian Biosearch, Perth, Australia) containing 10 $\mu\text{g/mL}$ Deoxyribonuclease 1 (DN-25, Sigma Chemical Co, St Louis, Mo) and filtered through a cell-strainer cap (Falcon, BD) to remove cell clumps. Gates were set by using concentration-matched isotype controls. Samples were read on a 6-color laser BD LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Inc, Ashland, Ore).

Isolation of thymic Treg, effector T, and autologous antigen-presenting cells for functional studies

Thymic Treg cells were isolated as described above; however, the permeabilization step was omitted, and additional CD8-PECy7 (1:5) was used for gating (BD Biosciences). Briefly, 60×10^6 thymocytes from each patient were thawed and a small aliquot (2×10^6 cells) was kept aside for CD3 depletion. The remaining thymocytes were stained with CD4-AF488 (1:5), CD25-allophycocyanin (1:2.5), CD127-phycoerythrin (1:10), and CD8-PECy7 (1:5) for 30 minutes on ice in the dark, washed, and filtered. Cells were sorted with a FACSAria (BD Biosciences) into 2 populations: $\text{CD8}^-\text{CD4}^+\text{CD25}^+\text{CD127}^{\text{low/-}}$ (Treg cells) and $\text{CD8}^-\text{CD4}^+\text{CD25}^-\text{CD127}^+$ (CD4^+ effector T cells). CD3 depletion was performed with magnetic CD3 Dynalbeads (Dynal Biotech, Invitrogen, Mulgrave, Victoria, Australia), according to the manufacturer's instructions. Cell purities were routinely greater than 90%.

Assessment of Treg cell function

Effector T cells ($\text{CD4}^+\text{CD25}^-\text{CD127}^+$; 25,000 cells per well) were cocultured with autologous CD3-depleted thymocytes (antigen-presenting cells;

50,000 cells per well) in the presence or absence of autologous Treg cells ($CD4^+CD25^+CD127^{lo/-}$; 25,000/50,000/75,000 cells per well) and cultured with or without SEB (200 ng/mL) to assess the ability of thymic Treg cells to suppress effector responses in the thymus and how this changes with age and atopic status. Forty-eight hours later, supernatants were collected (for cytokine detection), and cell pellets were resuspended in RNeasy Lysis Buffer for RNA extraction. Parallel coculture experiments were set up and processed for immunofluorescence to determine whether thymic $CD4^+$ T cells express IL-13 and whether they are regulated by Treg cells. For further details, see the Methods section in this article's Online Repository at www.jacionline.org.

Cytokine measurements

Levels of T_H1 (IFN- γ), T_H2 (IL-13), and immunoregulatory (IL-10) cytokines from supernatants were measured with Luminex Xmap (Luminex Corp, Austin, Tex), as previously described.¹⁴ The limit of detection for each cytokine was 3 pg/mL. Cytokine data are expressed as the change above unstimulated control values.

FOXP3 and IL2 mRNA expression determined by using RT-PCR

RNA was isolated from cell pellets with the RNeasy Mini kit (Qiagen, Clifton Hill, Australia) and reverse transcribed with the Omniscript RT kit (Qiagen) with oligo-dT (Promega, Madison, Wis) and Superscript (Geneworks, Hindmarsh SA, Australia). Intron-spanning primers for *FOXP3* and *IL2* were designed in house by using Primer Express Software (Applied Biosystems, Foster City, Calif). Reverse transcribed RNA samples were diluted 1:5 and quantified by means of real-time PCR with QuantiTect SYBR Green Master Mix (Qiagen) on the ABI PRISM 7900HT (Applied Biosystems). Melting and dissociation curve analyses were used to confirm the specificity of product amplification. Relative copy numbers were determined by using 10-fold serial dilutions of plasmid or PCR product standard curves and normalized to the endogenous reference gene ubiquitin-conjugating enzyme E2D2 (*UBC*).

Thymic stromal lymphopoietin immunocytochemistry

Thymic stromal lymphopoietin (TSLP)-positive cells were identified on paraffin-embedded tissue sections (4 μ m) by using a rabbit polyclonal antibody directed against TSLP (ab47973, 1:800; Abcam, Cambridge, Mass) with the EnVision Dual Link Detection System (Dako, Glostrup, Denmark) and visualized with Dako Liquid DAB+ Substrate-Chromogen. Slides were counterstained with Carazzi hematoxylin, dehydrated, and cover slipped in Ultramount permanent mounting medium (Dako). TSLP-positive cells appeared brown. For negative controls, the primary antibody step was omitted or an isotype control used. The total number of TSLP-immunoreactive cells was counted on 10 random nonoverlapping grids by using a Leica DM LB 100L microscope (Leica Microsystems, Wetzlar, Germany) with $\times 40$ magnification and an eyepiece graticule of 0.202 mm².

Incubation of thymic cultures with anti-TSLP

TSLP is an IL-7-like cytokine that triggers dendritic cell-mediated T_H2 -type inflammatory responses and is considered a master switch for allergic inflammation.^{15,16} In some cultures anti-TSLP antibody (10 μ g/mL; R&D Systems, Minneapolis, Minn) was added with Treg cells to examine the effect of TSLP on Treg cell suppressive function. Here, 75,000 Treg cells per well were used (1:3 ratio with effector cells), and *IL2* mRNA expression was measured in cell pellets, as described above.

Statistical analyses

Results are expressed as box-and-whisker plots. Horizontal bars indicate median values, boxes indicate interquartile ranges, and whiskers delineate maximum and minimum values. Groups were compared by using 2-tailed Mann-Whitney tests because data were nonnormally distributed. Statistical analyses were performed with SPSS version 18 (SPSS, Inc, Chicago, Ill) or

Prism version 5 (GraphPad Software, Inc, San Diego, Calif) software. Significance was set at a *P* value of less than .05.

RESULTS

Study population

To identify our target 20 atopic children, we recruited 62 children for allergy follow-up assessments, anticipating a prevalence of approximately 40% based on previous data from Australian children.¹⁷ The prevalence of atopy (allergen sensitization determined by skin reactivity) in this population was 36% (21/59). For each of the 20 atopic children (with suitable samples), 20 age-matched control subjects were identified from the remaining 38 nonatopic children. This achieved equal numbers (5 atopic and 5 nonatopic children) in each of 4 age groups (<6 months, 6-12 months, 1-5 years, and >5 years), enabling us to examine ontogeny of thymic Treg cell function in cross-sectional design and to assess for differences in atopic and nonatopic children. The patients' characteristics are shown in Table E1 in this article's Online Repository at www.jacionline.org. Although we selected on the basis of sensitization, it is notable that almost all children (18/20) also had diagnosed allergic disease and that none (0/20) of the nonatopic (negative SPT response) group had any disease, reinforcing that the clinical phenotypes used in this comparison are clearly distinct.

Thymic microenvironment is T_H2 skewed at birth: Atopy delays its maturation

The thymic microenvironment of both atopic and nonatopic children is T_H2 skewed during the early postnatal period (<6 months), with high production of IL-13 after PHA stimulation (see Fig E1 in this article's Online Repository at www.jacionline.org). In nonatopic children IL-13 production undergoes age-related suppression as their T_H1 (IFN- γ) and immunomodulatory (IL-10) cytokine production increases. Although the profile of T-cell maturation is similar in atopic children, their T_H2 downregulation and T_H1 maturation are significantly delayed compared with those seen in age-matched nonatopic subjects, and their IL-10 responses remain unchanged with age (see Fig E1). A similar cytokine profile was seen after SEB stimulation (data not shown).

Characterization and enumeration of Treg cells with age and atopic status

We detected an age-dependent increase in the proportion of thymic $CD4^+CD25^+CD127^{lo/-}FOXP3^+$ Treg cells (as a percentage of total thymocytes), suggesting Treg cell maturation in the thymus (Fig 1). In nonatopic neonates $CD4^+CD25^+CD127^{lo/-}FOXP3^+$ cells made up approximately 2% to 4% of total thymocytes (Fig 1, B, left panel) or 5% to 10% of mature $CD4^+CD8^-$ cells (Fig 1, B, right panel) and greater than 8% in children older than 5 years (Fig 1, A). Although a similar trajectory was seen in atopic children, the rate of maturation was significantly slower after the first 12 months compared with that seen in nonatopic children of the same age (Fig 1, B). Parallel age-dependent maturation in *FOXP3* mRNA expression was seen in nonatopic children (Fig 1, C). Atopic subjects had significantly reduced *FOXP3* mRNA expression from 6 months of age compared with that seen in age-matched nonatopic control subjects (*P* = .012; Fig 1, C).

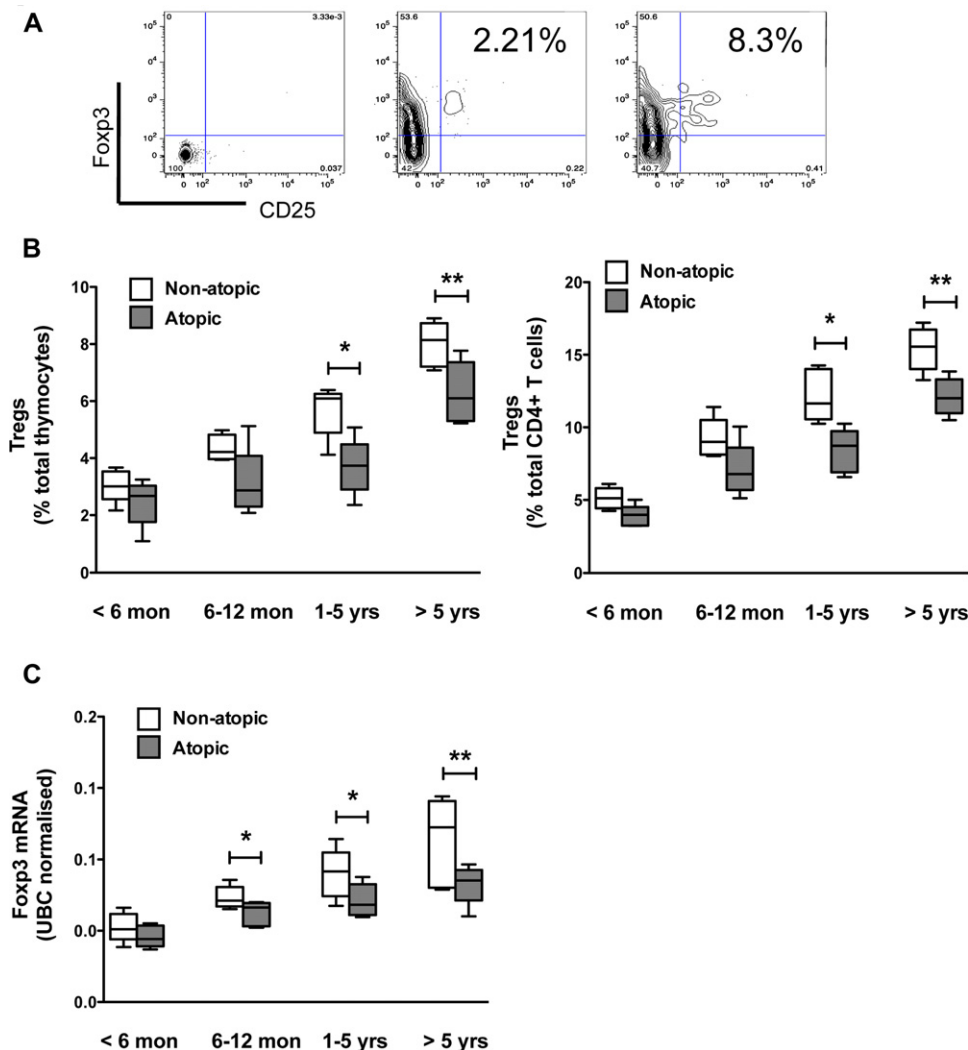


FIG 1. Enumeration of thymic Treg cells with age in atopic and nonatopic children. **A**, FOXP3⁺CD25⁺ Treg cell expression in the thymus from a neonate (middle panel) or a 6-year-old child (right panel). Total thymocytes were initially gated on CD4⁺ T cells and then on CD127^{lo/-} cells. The left panel shows staining with the isotype control in the thymus from the 6-year-old child. **B**, Percentage of Treg cells as the proportion of total thymocytes in the left panel and the proportion of total CD4⁺ T cells in the right panel. **C**, FOXP3 mRNA expression in nonatopic (white bars) and atopic (gray bars) children with age (n = 10 per age group). Results are expressed as box-and-whisker plots. *P < .05 and **P < .001 versus nonatopic age-matched control subjects.

Turnover of thymic Treg cells with age and atopic status

We observed a greater percentage of Treg cells undergoing turnover (CD4⁺CD25⁺CD127^{lo/-}) in older compared with younger children (see Fig E2 in this article's Online Repository at www.jacionline.org), supporting intrathymic Treg cell proliferation with age. At 5 years, almost 30% of all Treg cells in nonatopic thymic tissue were turning over compared with only 10% to 15% during the first 6 months of life (see Fig E2). This turnover was significantly ($P < .05$) impaired in atopic children after the first 6 months compared with that seen in age-matched nonatopic control subjects (see Fig E2). A similar proportion of CD25⁺ cells undergoing turnover was observed between older and younger nonallergic subjects ($5.25\% \pm 2.02\%$ vs $2.88\% \pm 1.47\%$ [mean $\pm$ SEM], n = 5, $P = .38$) and between atopic neonates and older

atopic children (data not shown). No difference in turnover of thymic effector T cells (CD4⁺CD127⁺Ki67⁺) was seen with age or between atopic and nonatopic age-matched control subjects (data not shown).

Function of thymic Treg cells with age and atopic status

Treg cell function was measured by capacity to suppress central effector responses in the thymus. Stimulation of effector T cells (CD4⁺CD25⁺CD127⁺) with SEB (or PHA) in the presence of autologous antigen-presenting cells (CD3⁺) significantly increased IL2 mRNA expression and IL-13 protein production at 48 hours from unstimulated control subjects (data not shown). Addition of CD4⁺CD25⁺CD127^{lo/-} Treg cells induced a dose-

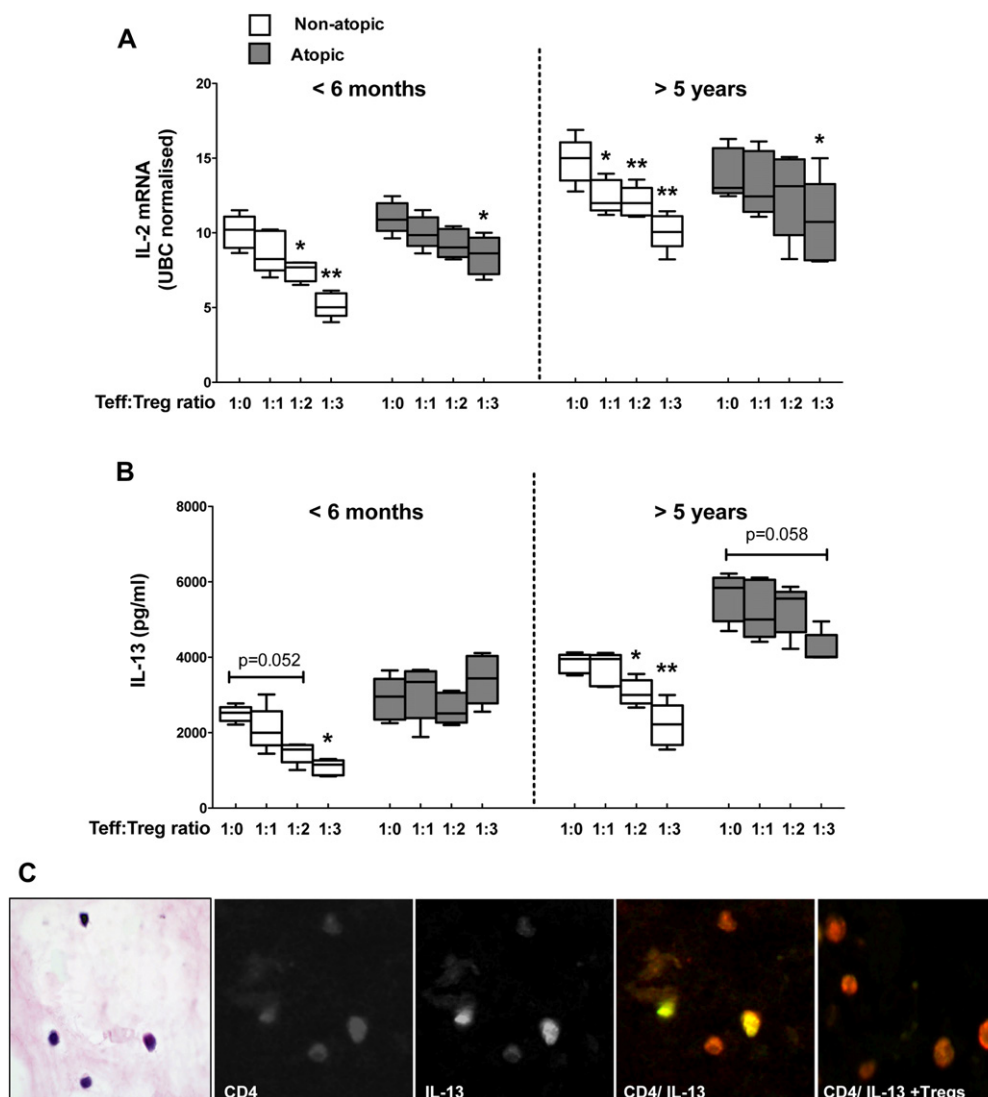


FIG 2. Thymic Treg cell function in atopic and nonatopic children. **A** and **B**, Effects of adding increasing numbers of Treg cells to effector T-cell (*Teff*) cultures on *IL2* mRNA expression (Fig 2, **A**) and IL-13 protein production (Fig 2, **B**) in nonatopic (white bars, $n = 10$) or atopic (gray bars, $n = 10$) children less than 6 months of age (left panel) or more than 5 years of age (right panel). Results are expressed as box-and-whisker plots. * $P < .05$ and ** $P < .001$ versus Treg cell ratio 1:0 (SEB stimulated without Treg cells). **C**, Immunofluorescence imaging showing individual channels in grayscale depicting staining for thymic CD4⁺ T cells (panel 2), IL-13⁺ cells (panel 3), and their colocalization (merge image in panel 4, with CD4 as red, IL-13 as green, and colocalization as yellow immunostaining), which is reduced with addition of Treg cells in nonatopic children (panel 5). Panel 1 is the hematoxylin and eosin stain of the same area used for immunofluorescence.

dependent reduction in *IL2* mRNA expression in nonatopic neonates (<6 months of age; Fig 2, **A**). Similar but more robust responses were seen in older nonallergic children. The suppressive effect in atopic children (neonates and those older than 5 years) did not reach significance for *IL2* suppression except when large numbers of Treg cells (1:3 ratio) were added (Fig 2, **A**). Addition of Treg cells reduced effector T-cell production of IL-13 in both groups of nonatopic children (Fig 2, **B**) but had no effect in atopic subjects, even at the highest dose of Treg cells (Fig 2, **B**). IL-13 expression by neonatal thymic CD4⁺ T cells is visually depicted in Fig 2, **C**. The number of CD4⁺IL-13⁺ cells was significantly reduced with addition of Treg cells in

nonallergic (from 7.3 ± 2.1 to 2.3 ± 0.4 cells per field [mean $\pm$ SEM], $n = 5$, $P = .032$) but not allergic (from 8.7 ± 3.5 to 7.9 ± 3.8 cells per field, $n = 5$) neonates.

Mechanism or mechanisms of thymic Treg cell maturation/suppression

TSLP, a cytokine produced by the epithelial cells in thymic Hassall corpuscles, has gained much interest as a key initiator of allergic diseases.^{18,19} TSLP directly activates TSLP receptor (TSLPR) on thymic DCs, and in mice TSLP-activated dendritic cells have been implicated in conversion of self-reactive T cells

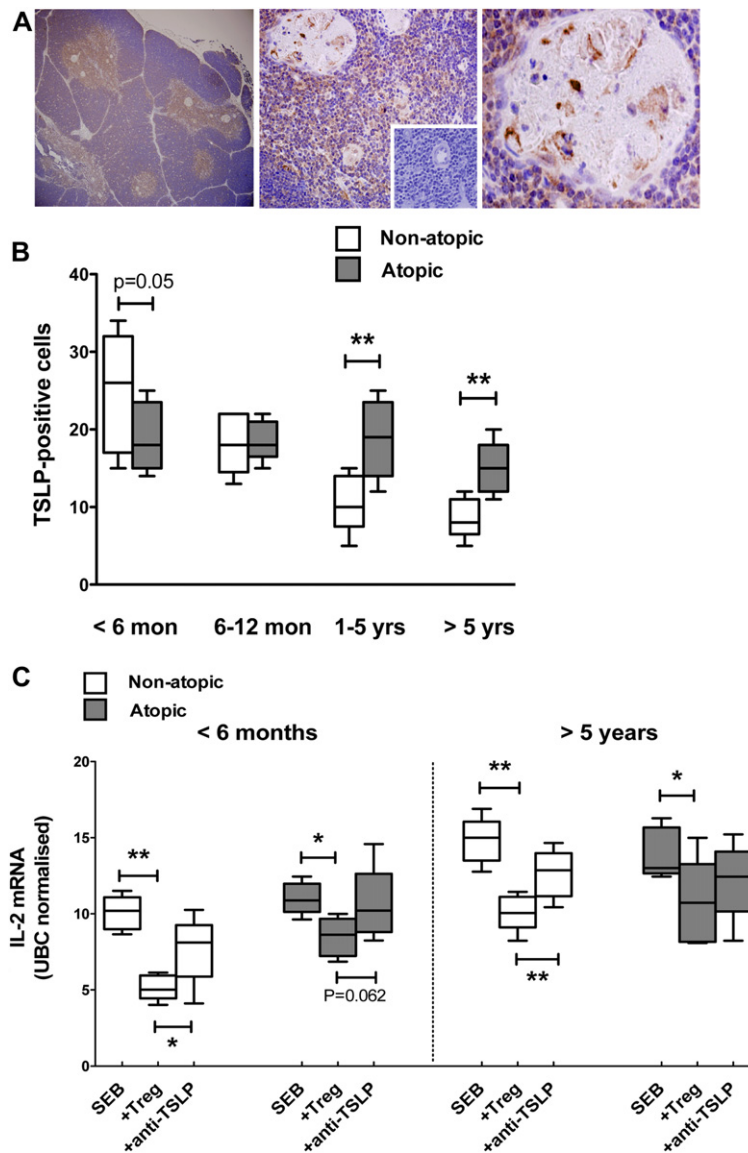


FIG 3. Maturation of TSLP with age and its effect on Treg cell suppression in atopic and nonatopic children. **A**, TSLP immunoreactivity in the medulla (left panel) and Hassall corpuscles (middle and right panels) of neonatal thymic tissue from nonatopic subject. The insert in the middle panel is stained with isotype control antibody. **B**, Number of TSLP-immunoreactive cells in nonatopic (white bars, $n = 20$) and atopic (gray bars, $n = 20$) children with age ($n = 10$ per age group). **C**, Effect of blocking TSLP on Treg cell-induced suppression of effector T-cell function in nonatopic (white bars, $n = 10$) or atopic (gray bars, $n = 10$) children less than 6 months of age (left) or more than 5 years of age (right). Results are expressed as box-and-whisker plots. * $P < .05$ and ** $P < .001$.

into Treg cells.^{18,19} We demonstrated strong and diffuse TSLP immunoreactivity in the medulla (where T-cell “education” occurs) and Hassall corpuscles of nonatopic neonatal thymus (Fig 3, A). At birth, TSLP immunoreactivity was greater in nonatopic compared with atopic subjects ($P = .05$; Fig 3, B). In nonatopic subjects the number of TSLP-reactive cells decreased with age and was very similar to the T_H2 maturation profile in the thymus seen in the same group of neonates (see Fig E1). TSLP immunoreactivity remained relatively unchanged from birth in atopic subjects (Fig 3, B) so that by 12 months of age, nonatopic children had significantly lower TSLP immunoreactivity compared with that seen in age-matched atopic control subjects (Fig 3, B). This

was also seen in the oldest group of children ($P = .0008$). Our data cannot distinguish whether this difference in TSLP immunoreactivity is due to sensitization in atopic subjects or that sensitization is due to increased TSLP levels.

Next we explored the role of TSLP in suppression of SEB-induced effector responses by Treg cells. Culturing thymocytes in the presence of TSLP blocking antibody reduced Treg cell suppressive function (*IL2* mRNA expression) and restored approximately 80% of T-cell effector function in nonatopic neonates and older nonatopic children (Fig 3, C). A similar trend was observed for nonatopic neonates ($P = .062$). Although anti-TSLP did not statistically restore effector function in older atopic

children, in 4 of 5 subjects it restored *IL2* mRNA expression similar to those seen in the absence of Treg cells (Fig 3, C).

DISCUSSION

Although many studies have demonstrated that the relative immaturity of many aspects of immune function are associated with allergic disease,²⁰⁻²² it has not been clear whether this extends to regulatory pathways. In this study, for the first time, we have shown that Treg cell function is developmentally regulated within the thymus and that there are early differences in Treg cell maturation and function in atopic compared with nonatopic children. Furthermore, consistent with our previous studies of the peripheral immune system,²² we have now demonstrated that the thymic microenvironment is similarly T_H2 -skewed in the early postnatal period and that it undergoes age-related suppression in favor of increasing T_H1 maturation. In addition to eosinophils being an important source of T_H2 cytokines in the neonatal thymus,¹³ here we show that thymic $CD4^+$ T cells contain IL-13 and that neonatal thymic Treg cells can directly suppress their effector function. This suppression is more effective in nonatopic compared with atopic children.

This is the first study to document the presence of functional Treg cells in human neonatal thymus and that their function is significantly delayed in atopic children. These Treg cells have strong dose-dependent suppressive effects on autologous effector T-cell responses in the thymus. However, in the atopic group the highest titration of neonatal Treg cells was required to significantly inhibit *IL2* mRNA expression but still failed to downregulate neonatal IL-13 protein production. A similar delay in the Treg cell suppressive effect was seen in older atopic children, although the magnitude of their responses to SEB and PHA was higher compared with neonatal responses. These data are in support of previous studies showing that human $CD4^+CD25^{++}$ cells mature in the thymus in which they acquire CD45RA and CD62 ligand expression.²³ Suppression of allergen-induced cytokine production by thymic Treg cells has been shown in response to myelin oligodendrocyte glycoprotein, tetanus toxoid, and β -lactoglobulin, confirming the presence of antigen-specific Treg cells in the human thymus.²⁴ Together, these results suggest that there are presymptomatic differences in thymic Treg cell function between children with allergic sensitization (and are therefore at higher risk of allergic disease) and those who remain nonatopic. Moreover, our data suggest that neonatal Treg cells are already functional in the thymus and behave like peripheral Treg cells, which are impaired in high-risk infants²⁵ and children with established allergic disease.¹¹

One of the mechanisms by which thymic Treg cells exert their suppressive function is through a contact-dependent mechanism related to the combined action of surface CTLA-4 and TGF- β 1 leading to inhibition of the IL-2 receptor α chain (CD25) on target T cells.²⁶ We can speculate that lack of CD25 inhibition in thymic T cells or reduced expression of CTLA-4, TGF- β 1, or both in the thymus might be responsible for the reduced Treg cell function we observed in atopic children compared with that seen in age-matched nonatopic control subjects. Indeed, we showed a direct effect of Treg cells on IL-13 production from thymic $CD4^+$ T cells (Fig 2, C) in nonatopic but not atopic neonates.

Another critical factor in Treg cell development is TSLP, the expression of which is increased in allergic patients.²⁷ Maturation of thymic Treg cells requires activation of the TSLPR/IL-7

receptor α receptor complex by TSLP,²⁸ and disruption of TSLP signaling impairs intrathymic generation of Treg cells and their functional maturation.¹⁵ We have shown inhibition of thymic Treg cell function in children with addition of anti-TSLP. This effect was more pronounced in nonatopic compared with atopic neonates. The proposed mechanism or mechanisms in TSLP-induced regulation of Treg cell function involves direct conversion of reactive $CD4^+CD25^-$ thymic T cells into $CD4^+CD25^+FOXP3^+$ Treg cells²⁹ and their regulation of IL-10 production.³⁰ We now know that human pulmonary Treg cells express TSLPR and that TSLP can directly modulate Treg cell function.³⁰ Here we show, for the first time, that this regulation occurs in the thymuses of children and that the sensitivity of Treg cells to TSLP is dependent on the atopic status of the child. Furthermore, we have shown thymic TSLP immunoreactivity to be at its highest levels in neonates (Fig 3, B), and our neutralization experiments (Fig 3, C) confirm its importance in thymic Treg cell function in early life. In contrast to its beneficial role in the thymus, in the periphery TSLP is a key initiator of T_H2 -driven allergic airway inflammation.¹⁹ TSLP is involved in driving a classic T_H2 bias at the human maternal-fetal interface through instructing $CD4^+$ T cells to produce T_H2 cytokines.³¹ More recently, a rare population of human IL-4-producing T_H2 cells has been shown to express TSLPR in a proallergenic milieu and might therefore directly amplify T_H2 responses.³² We might speculate that the high TSLP immunoreactivity observed in the neonatal thymus might be an important factor in maintaining the early T_H2 microenvironment we see in the thymus; however, this remains to be directly proved.

This study builds on our previous work with thymic tissue from young children.¹³ To examine Treg cell ontogeny, ideally, we would have liked to study thymic Treg cell function longitudinally; however, this was not possible as a result of thymectomy during cardiac surgery. One of the main potential confounders in this study is the role of thymectomy in the development of allergic disease. The consensus in the literature is that although thymectomy does result in a lower proportion of total lymphocytes, Treg cells appear to be less affected than other subsets,³³ and importantly, their function is unaffected.³⁴ Long-term follow-up of thymectomized patients suggests that peripheral T-cell homeostasis is maintained by extrathymic turnover of existing naive T cells in the periphery,³⁵ and these patients do not have compromised immune function.³⁶ On the basis of these studies, we would not expect the lack of thymus to enhance the development of allergy. Indeed, the prevalence of allergic sensitization in our cohort is very similar to that seen in other cohorts and the local general population.

In summary, our results support the early T_H2 -driven microenvironment in the thymus and postnatal maturation of local immunity from T_H2 to T_H1 responses, which is similar to changes previously described in the periphery. The thymus is a rich source of Treg cells, which increase in number and function with age. Thymic Treg cells have strong suppressive ability, even in neonates (with a direct effect on IL-13-producing $CD4^+$ cells), and their function is TSLP dependent (particularly in nonatopic children). Our results support developmental changes in regulatory function in the thymus and early differences in those children with allergic sensitization. This finding is critical in understanding early events that lead to immune dysregulation in infancy and mechanisms that might predispose to allergic disease. Finally, our study supports the use of the thymus as a useful tool for exploring the development of immune-regulatory function in young children in whom isolation of peripheral Treg cells can be difficult

from small blood volumes. Further elucidation of the mechanisms involved in failure of immune regulation could ultimately help in the development of strategies to optimally modify these pathways for disease prevention.

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Key messages

- Impaired immune regulation in early life has become a leading hypothesis to explain the escalating rate of allergic disease.
- The thymus offers a novel opportunity to study Treg cell function.
- We have documented the normal development of thymic Treg cells from birth to puberty and have shown the importance of TSLP in the maturation of this response.
- Children who go on to have allergic sensitization (atopy) show functional differences (delay) in the development of these critical pathways.

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METHODS

Clinical definitions

The main allergic outcomes were eczema, IgE-mediated food allergy, and sensitization. A diagnosis of eczema was based on typical skin lesions responsive to topical steroids. IgE-mediated food allergy was defined as a history of immediate (within 60 minutes) symptoms after contact with and/or ingestion of food (eg, egg, dairy, or nut) and a positive SPT response to that food. Symptoms of acute food allergy included skin reactions (hives, rash, or swelling) and/or respiratory symptoms (cough, wheeze, and stridor) and/or gastrointestinal symptoms (abdominal pain, vomiting, and loose stools) and/or cardiovascular symptoms (collapse). Diagnosis of asthma was based on a history of recurrent wheeze with responsiveness to bronchodilator medications.

Assessment of Treg cell function

Parallel coculture experiments ($n = 3$) were set up to determine (1) whether thymic $CD4^+$ T cells express IL-13 and (2) whether this expression

is regulated by Treg cells. Here, cell pellets were pooled and resuspended in 0.5 mL of plasma (Serviotrol A; Excel Laboratories, Perth, Australia) and 0.5 mL of Thrombin Reagent (Dade Behring, Siemens Healthcare, Marburg, Germany) before being placed in a 37°C oven for 10 minutes and allowed to clot. The clot was made into paraffin blocks and processed for immunofluorescence. Sections were stained with CD4 mAb (1:100; Pathology Products, Dako Australia) and polyclonal IL-13 antibody (1:25; Santa Cruz Biotechnology, Santa Cruz, Calif). $CD4^+$ cells were visualized with goat anti-mouse antibody labeled with Texas Red (1:100) and IL-13 with goat anti-rat conjugated to fluorescein isothiocyanate (1:100, Dako). Control sections were stained with a single color or their respective isotype controls, and background autofluorescence was not significant. Colocalization of $CD4^+$ T cells with IL-13 was identified as yellow immunostaining with a Nikon Eclipse Ti-U inverted microscope (Melville, NY) with $\times 40$ magnification.

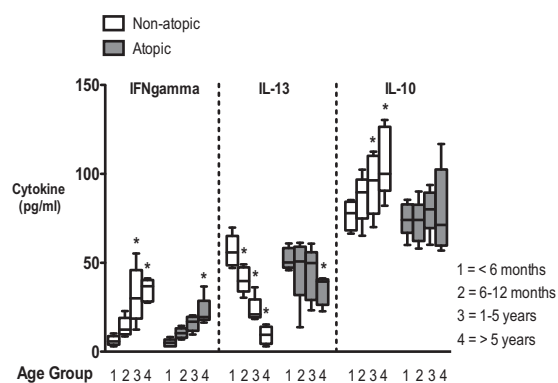


FIG E1. Thymic cytokine maturation in atopic and nonatopic children. Cytokine expression in nonatopic (*white bars*) or atopic (*gray bars*) children less than 6 months (group 1: $n = 10$), 6 to 12 months (group 2: $n = 10$), 1 to 5 years (group 3: $n = 10$), or greater than 5 years (group 4: $n = 10$) after PHA stimulation. Results are expressed as box-and-whisker plots. * $P < .05$ versus group 1 for each cytokine.

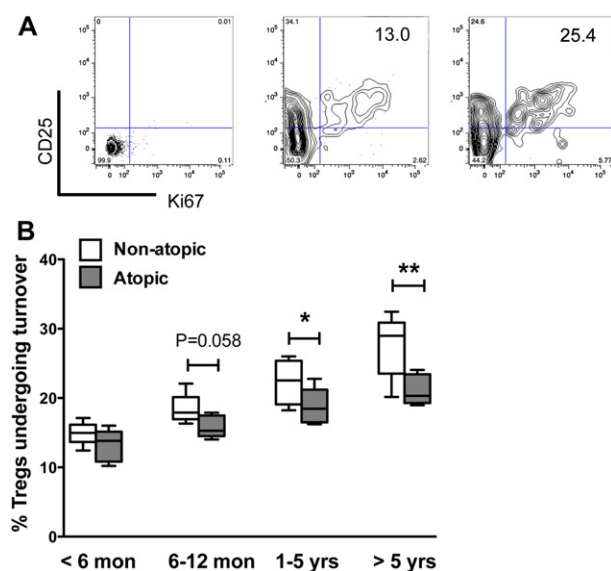


FIG E2. Turnover of thymic Treg cells with age in atopic and nonatopic children. **A**, $CD4^+CD25^+CD127^{lo/-}Ki67^+$ Treg cell proliferation in the thymus from a neonate (*middle panel*) or 6-year-old child (*right panel*). Total thymocytes were initially gated on $CD4^+$ T cells and then on $CD127^{lo/-}$ cells. The *left panel* shows staining with the isotype control in the thymus of 6-year old child. **B**, Percentage of Treg cells ($CD4^+CD25^+CD127^{lo/-}Ki67^+$ cells) undergoing turnover in nonatopic (*white bars*, $n = 20$) and atopic (*gray bars*, $n = 20$) children with age ($n = 10$ per age group). Results are expressed as box-and-whisker plots. * $P < .05$ and ** $P < .001$ versus nonatopic age-matched control subjects.

TABLE E1. Characteristics of the study population

Characteristics	Nonatopic (n = 20)	Atopic (n = 20)	Significance, <i>P</i> value
Sex			
Male	12/20 (60%)	11/20 (55%)	.68
Female	8/20 (40%)	9/20 (45%)	.76
Any allergic disease	0/20 (0%)	18/20 (90%)	.028
Atopic dermatitis	0/20 (0%)	11/20 (55%)	.036
Asthma	0/20 (0%)	2/20 (10%)	NA
Food allergy (IgE-mediated)	0/20 (0%)	5/20 (25%)	.047
Sensitized	0/20 (0%)	20/20 (100%)	<.001
House dust mite	0/20 (0%)	11/20 (55%)	<.001
Grass pollens	0/20 (0%)	3/20 (15%)	<.001
Egg	0/20 (0%)	2/20 (10%)	<.001
Peanut	0/20 (0%)	1/20 (5%)	<.001
Multiple sensitizations	0/20 (0%)	3/20 (15%)	<.001

Values are presented as number (percentages). Significant values are shown in boldface.

NA, Not available.