Association between CD4$^+$CD25^{high} T cells and atopy in children

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Background: There is evidence that CD4$^+$CD25^{high} T-regulatory cells are important for establishing tolerance to allergens, but information in children is limited.
Objective: To test the hypothesis that greater numbers and function of CD4$^+$CD25^{high} T cells are associated with a reduced risk of childhood allergies and wheezing.
Methods: A cohort of 151 six-year-old children from atopic families was analyzed for peripheral blood CD4$^+$CD25^{high} and CD4$^+$CD25^{int} T cells by flow cytometry and for clinical and immunologic correlates of atopy. The associations between these variables were assessed by regression analysis.
Results: Factors positively associated with % CD4$^+$CD25^{high}/CD4 T cells were male sex, number of positive allergen-specific IgE tests, total IgE, season, and 1-month average total pollen count preceding blood draw. The percentage of CD4$^+$CD25^{high}/total CD4 T cells did not correlate with induced cytokine production, and correlated negatively with suppressive capacity of CD4$^+$CD25$^+$ T cells ($r = -0.45$; $P = .034$). The percentage of CD4$^+$CD25^{int}/CD4 T cells was 54% higher in pollen-sensitized children compared with nonsensitized children in spring ($P = .023$ for interaction), and correlated positively with IL-5, IL-10, and IL-13 ($P \leq .001$ for all).
Conclusion: Our findings suggest that blood CD4$^+$CD25^{high} cells are a mixture of activated and regulatory T cells, and that these cells could be seasonally regulated by environmental factors such as pollen exposure.
Clinical implications: Seasonal increases in CD4CD25^{high} expression in children with allergy may represent systemic immune activation caused by pollen exposures. (J Allergy Clin Immunol 2007;120:177-83.)

Key words: *Regulatory CD4$^+$CD25$^+$ T cell, activated T cell, seasonality, atopy, pollen sensitization, cytokine, wheezing, atopic dermatitis, sex, child*

The activation of T_H2 cells is considered to be crucial in allergic sensitization.[1] T_H2 cells produce cytokines, such as IL-4, IL-5, and IL-13, that mediate IgE synthesis and eosinophilic inflammation and altogether contribute to airway hyperresponsiveness. Why atopic individuals develop T_H2 responses to allergens whereas nonatopic subjects do not is incompletely understood. One possible explanation may be the influence of T-regulatory (T_{reg}) cells that serve to promote tolerance to allergic and other inflammatory stimuli. CD4$^+$CD25$^+$ T_{reg} cells are generated in the thymus and reside in the blood and other peripheral lymphoid tissues at a frequency of 5% to 10% of total CD4$^+$ cells.[2] It has been suggested that pollen exposure increases the number of CD4$^+$CD25$^+$ T cells in subjects with pollen allergy, and that allergy may be associated with defective function of regulatory CD4$^+$CD25$^+$ T cells, which are unable to suppress pollen-induced activation of CD4$^+$CD25$^-$ T cells during pollen seasons.[3-5]

One limitation of these studies is that activated and regulatory CD4$^+$ T cells have overlapping expression of CD25, despite quite different functional capabilities. The 2 basic features of CD4$^+$CD25$^+$ T_{reg} cells are an anergic response to antigen stimulation and the ability to suppress proliferation of CD4$^+$CD25$^-$ T cells in a contact-dependent fashion.[5-10] Selection of the CD4$^+$CD25^{high} subset of CD4$^+$CD25$^+$ T cells has been suggested to minimize the coisolation of contaminating activated CD4$^+$ T cells. Furthermore, forkhead/winged helix transcription factor transcription factor (FOXP3), which is predominantly expressed by CD4$^+$CD25$^+$ T cells, appears to be a more specific marker for CD4$^+$CD25$^+$ T_{reg} cells.[11,12]

In general, the knowledge on the role and suppressive effect of CD4$^+$CD25$^+$ T_{reg} cells in atopic subjects is still limited, especially in children, and somewhat

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Abbreviations used
COAST: Childhood Origins of ASThma
FOXP3: Forkhead/winged helix transcription factor transcription factor
SPT: Skin prick test
T_{reg}: T-regulatory

contradictory.[10] To test the hypothesis that T_{reg} cells reduce the risk of developing allergy and wheezing in childhood, we compared the number and function of $CD4^+CD25^{high}$ T cells to several clinical and immunologic factors in a well characterized group of children born to atopic families.

METHODS

Study subjects and study design

Originally, 289 subjects were enrolled in the Childhood Origins of ASThma (COAST) study at birth, and 257 were followed prospectively for at least 6 years. Of these 257 children, flow-cytometric analysis of PBMCs was performed for 151 children at the scheduled 6-year visit between February 2005 and March 2006. Within this time frame, all COAST participants with enough PBMCs ($2\text{-}4 \times 10^6$ cells after COAST cytokine secretion assays were completed) for the flow-cytometric analysis at the 6-year visit (close to the birthday) were included in this study. Flow-cytometric data were correlated with other clinical and laboratory data obtained at age 5 to 6 years.

To be eligible, each of the COAST children was required to have 1 or both parents with allergic sensitization (1 or more positive aeroallergen skin tests) and/or asthma (by history), ≥37 weeks of gestation, and be otherwise healthy. Details of study population and design have been described previously.[13] This study was approved by University of Wisconsin Human Subjects Committee and commenced only after obtaining informed consent.

Flow cytometry

Within 24 hours of blood draw, PBMCs were isolated and were then stained with antibody/conjugate: CD3/peridinin chlorophyll protein, CD4/allophycocyanin-7, CD25/allophycocyanin, CD62L/phycoerythrin-cyanine 5, CD45RA/fluorescein isothiocyanate, and glucocorticoid-induced TNF receptor–related gene/phycoerythrin (BD Biosciences, San Jose, Calif; see this article's Methods in the Online Repository at www.jacionline.org). Cells were analyzed on a LSR II (3-laser, 11-color benchtop flow cytometer; BD Biosciences) within 48 hours of blood draw. $CD3^+CD4^+CD25^-$ cells were determined by comparison with cells stained with an isotype control.

Beginning in March 2006, PBMC samples (n = 32; age 6-7 years) were analyzed for intracellular FOXP3 expression after the surface staining described. After cells were fixed and permeabilized (BioLegend, San Diego, Calif), they were incubated with Alexa Fluor 488 conjugated antihuman FOXP3 according to the manufacturer's instructions (BioLegend) and analyzed on a LSR II immediately after the staining procedure. $CD4^+CD25^+FOXP3^+$ cells were determined by comparison with cells stained with an isotype control.

Depletion of $CD4^+CD25^+$ T cells

An antibody-coated microbead isolation procedure was used for the depletion of $CD4^+CD25^+$ T cells (Miltenyi Biotec Inc, Auburn, Calif; see this article's Methods in the Online Repository at www.jacionline.org). This depletion protocol decreased mean $CD4^+CD25^{high}$ T-cell count by 62% (undepleted vs $CD4^+CD25^+$ depleted PBMCs, 8.7% vs 3.4%; n = 7; Fig 1, *A*) and increased mean anti-CD3/anti-CD28–induced proliferation by 17%.

Proliferation assay

To obtain a functional measure of T_{reg} activity, we used the proliferation suppression assay as described by Taams et al,[14] with modifications (see this article's Methods in the Online Repository at www.jacionline.org). Suppressive activity of $CD4^+CD25^+$ cells was estimated by dividing proliferative responses of CD4CD25-depleted PBMCs to responses by the same cells that had been repleted by adding back $CD4^+CD25^+$ cells in a 1:4 ratio. Larger indices represent more $CD4^+CD25^+$ suppressive capacity.

Total IgE and allergy tests

Total and allergen-specific IgE for birch, grass mix, ragweed, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria alternata*, cat, dog, cockroach, egg, and peanut were analyzed in 146 subjects by UniCAP 100^E as described previously.[15] The sensitivity for detection of total IgE was 2 kU/L. Allergen-specific IgE values of ≥0.35 kU/L were considered positive.

Skin prick testing (Multi-Test II; Lincoln Diagnostics, Decatur, Ill) was performed in 143 subjects for eastern tree mix, grass mix, weed mix, ragweed, *Alternaria alternata*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, dog epithelium, cat hair, and American/German cockroach (reagents and controls obtained from Greer Laboratories, Lenoir, NC). Tests were regarded as positive when the mean diameter of the wheal (half the sum of the largest diameter and its perpendicular measurement) was 3 mm or greater.

Immunologic studies

Specimens of peripheral blood at age 6 years and cytokine responses were performed as previously described.[15,16] PBMCs were incubated with phytohemagglutinin (5 μg/mL) or medium alone, and supernatant fluids collected 2 days later were analyzed for IFN-γ, IL-5, IL-10, and IL-13 by ELISA (Pharmingen, San Diego, Calif). The sensitivities of the ELISA were as follows: IFN-γ, 3.1 pg/mL; IL-5, 1.9 pg/mL; IL-10, 7.8 pg/mL; and IL-13, 3.1 pg/mL.

Pollen measurements

Pollen sampling was conducted at the University of Wisconsin School of Medicine and Public Health using Rotorod Samplers (Sampling Technologies, Minnetonka, Minn) in accordance with standards outlined by the Aeroallergen Monitoring Network of the American Academy of Allergy, Asthma, and Immunology (Milwaukee, Wis).[17]

Clinical definitions

Sensitization was defined either by a positive allergen-specific IgE test result at age 6 years or by a positive allergen-specific skin prick test (SPT) result at age 5 years or later. Pollen sensitization was defined as a positive IgE or SPT result for birch, eastern tree mix, grass mix, weed mix, or ragweed. Atopic dermatitis at age 6 years was defined by a parental report of physician-diagnosed atopic dermatitis. Wheezing history in the 6th year of life was documented by questionnaires at the 6th year protocol-scheduled visit that asked the parent whether the child had ever wheezed during the past year.

Statistics

Linear regression models were used to assess the relationship of clinical and seasonal factors with log-transformed proportions of

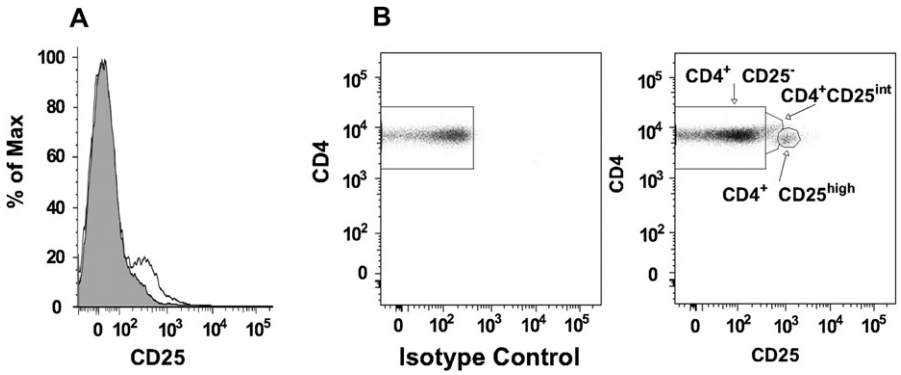

FIG 1. A, The magnetic depletion of $CD4^+CD25^+$ T cells decreased mean $CD4^+CD25^{high}$ T-cell count of $CD4^+$ T cells by 62%: undepleted *(white)* vs $CD4^+CD25^+$ depleted PBMCs *(gray)*, 8.7% vs 3.4%; n = 7. **B**, $CD4^+CD25^-$ T cells were determined by comparison with cells stained with an isotype control, and $CD4^+CD25^{high}$ T cells were identified visually as a distinct population with slightly lower CD4 expression compared with the $CD4^+CD25^{int}$ and $CD4^+CD25^-$ fractions.

T-cell subsets, in both univariate and multivariate models. Clinical and seasonal factors included sex; sensitization status grouped as pollen, other, or no sensitization; number of positive test results of specific IgE and SPTs; total IgE; active atopic dermatitis; active wheezing illness; season grouped as quarters of year; any controller medication (any on-demand or continuous medication for allergy or wheezing excluding creams); and continuous inhaled corticosteroid medication. Regression coefficients are back-transformed to represent percent change in the mean proportion of T-cell subsets. Associations between immunologic factors and T-cell subsets were assessed using Pearson or Spearman rank correlation coefficients when appropriate. A nominal *P* value of .05 was regarded as statistically significant.

RESULTS

The study population was composed of 151 children who had blood samples analyzed by flow cytometry at age 6 years. There were slightly more boys (59%; 89/151) than girls (see this article's Table E1 in the Online Repository at www.jacionline.org). More than half of the children were sensitized defined as either a positive allergen-specific IgE test result at age 6 years (any, 48%, 70/146; pollen, 20%, 29/146) or a positive allergen-specific SPT result at age 5 years or later (any, 47%, 67/143; pollen, 22%, 32/143). Overall, 30% (45/151) had pollen sensitization, and 27% (40/151) of children were sensitized to allergens other than pollen. Thirty-six percent (54/151) had active atopic dermatitis within the past year, and 21% (32/149) wheezed during the 6th year of life. Use of controller medication for allergic or wheezing illnesses was low (11%; 14/133).

Associations with CD4CD25 T-cell phenotype

The definition of $CD3^+CD4^+CD25^{high}$ cells was established *a priori*, and the cell populations were identified by a technician blind to protocol outcomes. Gates were drawn to capture a distinct population with high CD25 expression and slightly lower CD4 expression compared with other cells (Fig 1, *B*). $CD3^+CD4^+CD25^{int}$ was determined as those cells expressing levels of CD25 between that of the $CD3^+CD4^+CD25^-$ and $CD3^+CD4^+CD25^{high}$ populations (Fig 1, *B*). By univariate analysis (Table I), the percentage of $CD4^+CD25^{high}$/CD4 T cells (median, 5.6%; interquartile range, 4.8-6.8) was associated with male sex, number of positive specific IgE test results, total IgE, and season. In addition, there was a trend toward a positive association between % $CD4^+CD25^{high}$/CD4 T cells and the number of positive SPT results. In contrast, the percentage of $CD4^+CD25^{int}/CD4^+$ T cells (median, 4.3%; interquartile range, 3.0-5.9) was not significantly associated with any of these factors, although there was a trend in univariate models toward a positive association with total IgE.

In multivariate models (Table I), the associations with total IgE and season remained significant. The percentages of $CD4^+CD25^{high}$/CD4 T cells and % $CD4^+CD25^{int}/CD4^+$ T cells were loosely correlated ($r = 0.19$; $P = .020$). Finally, % $CD4^+CD25^{int}/CD4^+$ T cells were weakly associated with total IgE and inversely correlated with the number of positive allergen-specific IgE test results (Table I).

Relationship of CD25 and FOXP3 expression

Flow-cytometric analysis of FOXP3 was performed on a subset of samples beginning March 2006 (Fig 2). On average, 2.9% of CD4 cells expressed FOXP3 (n = 32). The percentage of FOXP3-expressing cells increased with expression of CD25 ($P < .0001$): 0.8% in $CD4^+25^-$ cells, 9.3% in $CD4^+25^{int}$ cells, 24% in $CD4^+25^{high}$ cells, and 88% in $CD4^+25^{brightest}$ cells (defined as cells with the top 2% of CD25 expression). Compared with $CD4^+25^{int}$ cells, $CD4^+25^{high}$ cells had greater CD62L (93% vs 79%; n = 31) and CD45RA (56% vs 23%; n = 19) expression and less glucocorticoid-induced TNF receptor–related gene (46% vs 59%; n = 19; $P < .0001$ overall). There was a significant negative correlation between total IgE and % FOXP3/$CD4^+25^{brightest}$ cells ($r = –0.42$; $P = .039$) and a negative trend with % FOXP3/$CD4^+25^{high}$ cells ($r = –0.39$; $P = .062$). The correlation between total

TABLE I. Clinical or seasonal factors associating with $CD4^+CD25^{high}$ and $CD4^+CD25^{int}$ T-cell counts*

		Percent $CD4^+CD25^{high}$/CD4 T cells						Percent $CD4^+CD25^{int}$/CD4 T cells					
		Univariate			Multivariate n = 120			Univariate			Multivariate n = 120		
Factor	n	Percent effect†	95% CI	*P* value	Percent effect	95% CI	*P* value	Percent effect	95% CI	*P* value	Percent effect	95% CI	*P* value
Male sex	151	11	1.0-21	.029	7.6	−2.3-18	.14	12	−4.4-31	.16	4.0	−15-25	.67
Any sensitization	151	6.8	−2.4-17	.15				7.5	−8.1-26	.36			
Sensitization group‡	151			.11			1.00			.61			.54
Pollen vs no sensitization		11	0.4-24		2.1	−11-13		9.6	−8.9-32		8.1	−14-36	
Other vs no sensitization		1.7	−8.9-14		0.4	−15-18		5.3	−13-27		19	−12-61	
No. of positive test results													
Specific IgE	146	2.0	0.0-4.0	.048	−2.1	−5.5-1.2	.21	0.6	−2.8-4.1	.74	–7.9	–14-1.6	.013
SPT	143	1.8	−0.0-3.8	.056	0.7	−2.2-3.7	.63	0.1	−3.0-3.4	.94	2.8	−2.8-8.6	.33
Log_{10}total IgE (per doubling)	146	3.6	1.5-5.8	.0007	5.6	2.3-9.0	.0007	3.1	−0.5-7.0	.093	6.5	0.3-13	.038
Atopic dermatitis	151	3.2	−6.1-13	.51	1.3	−7.7-11.3	.78	4.1	−12-23	.62	3.2	−13-23	.73
Wheezing	151	−6.5	−16-4.1	.22	−3.8	−19-14	.65	−8.6	−25-11	.35	−11	−36-24	.49
Season§	151			<.0001			<.0001			.95			.87
Spring vs winter		31	17-46		29	15-44		−3.7	−22-19		−7.0	−25-16	
Summer vs winter		8.7	−2.7-21		1.3	−9.9-14		−0.1	−19-23		−5.2	−24-18	
Fall vs winter		−2.6	−14-9.9		−5.0	−17-8.7		3.6	−16-30		1.8	−21-31	
Any controller medication	133	−9.9	−23-4.9	.18	−16	−33-3.9	.10	1.9	−22-33	.89	12	−25-69	.58
Continuous inhaled corticosteroid	133	−1.4	−20-22	.89	1.2	−21-29	.92	−3.4	−33-39	.85	−26	−53-17	.19

*Analysis performed using generalized linear regression model.
†Percent change in mean percent of $CD4^+CD25^{high}$ and $CD4^+CD25^{int}$ cells relative to $CD4^+$ T cells associated with a unit change in the each factor. Estimates are based on linear regression of log-transformed responses.
‡Grouped as no, pollen (excluding molds) and other sensitization. Pollen and other sensitization groups are compared to no sensitization group.
§Seasons equal quarters of year.

IgE and % FOXP3/CD4 was not significant (r = –0.24; P = .23; n = 24).

Interactions between allergy status and season

We considered that the spring increase in $CD4^+CD25^{high}$ T cells could be influenced by pollen allergy, but the presence of pollen-specific IgE did not significantly affect the seasonal variation of % $CD4^+CD25^{high}$ cells (P = .21; Fig 3, *A*). However, % $CD4^+CD25^{int}$ cells varied by sensitization group at different seasons (P = .023 in multivariate models). In spring, pollen sensitized children had with 54% higher % $CD4^+CD25^{int}$ cells than nonsensitized children (95% CI, 0.1, 139). In contrast, children with pollen allergy children had 63% lower % $CD4^+CD25^{int}$ compared with children with nonpollen allergies in the wintertime (95% CI, –142, –10; Fig 3, *B*).

We next investigated the association between T-cell counts and 1-month average total pollen count preceding blood draw (Fig 4). By univariate analysis, the preceding pollen count was strongly associated with % $CD4^+CD25^{high}$ cells (0.10% increase per one grain/m^3 of air; 95% CI, 0.050, 0.15; P = .0002), but not % $CD4^+CD25^{int}$ cells. By multivariate analysis, this relationship persisted after controlling for sex, sensitization status (pollen, other, or no sensitization), number of positive test results of specific IgE and SPTs, total IgE, active atopic dermatitis, active wheezing illness, any controller medication, and continuous inhaled corticosteroid medication (0.10% increase per 1 grain/m^3 of air, 95% CI 0.050, 0.15; P = .0009). In this model, the effect of pollen count on % $CD4^+CD25^{high}$/CD4 T cells varied by sensitization status (P = .016 for interaction). The relationship was present in children who were sensitized to other allergens but not pollen (0.22% per 1 grain/m^3 of air, 95% CI 0.11, 0.34), and in children who were sensitized to pollen (0.11% per 1 grain/m^3 of air, 95% CI, 0.02, 0.20), but absent in nonsensitized children (0.01% per 1 grain/m^3 of air, 95% CI, –0.07, 0.10).

CD4CD25 T-cell functional capacity

We next compared circulating number of cell subsets to regulatory cell functional capacity, as well as cytokine production from mitogen-stimulated PBMCs. The percentage of $CD4^+CD25^{high}$/$CD4^+$ T cells correlated negatively with $CD4^+CD25^+$ suppressive capacity (r = –0.45; P = .034; Fig 5). Suppressive capacity of $CD4^+CD25^+$ cells was not related to the presence of any allergic sensitization (P = .51), sensitization classified by pollen or

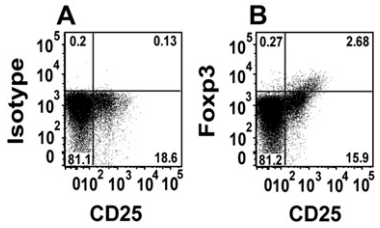

FIG 2. Intracellular FOXP3 expression on $CD3^+CD4^+CD25^+$ cells. PBMCs were stained with CD3/Pacific Blue, CD4/allophycocyanin-7, CD25/phycoerythrin, and CD62L/phycoerythrin-cyanine 5 and then intracellular-stained with an Alexa Fluor 488 isotype control **(A)** or Alexa Fluor 488 antihuman FOXP3 **(B)**.

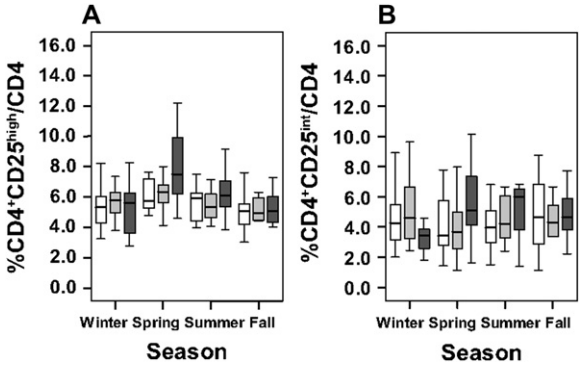

FIG 3. Seasonal changes in % $CD4^+CD25^{high}$/CD4 T cells **(A)** and % $CD4^+CD25^{int}$/CD4 T cells **(B)** grouped according to sensitization status (none, *white*; other than pollen, *light gray*; pollen, *dark gray*). In spring, pollen-sensitized children had a 54% higher $CD4^+CD25^{int}$/CD4 T-cell count than nonsensitized children (95% CI, 7.3, 161) by multivariate analysis ($P = .023$ for interaction). Sample sizes for winter, spring, summer, and fall seasons, respectively, are 49, 34, 40, and 28.

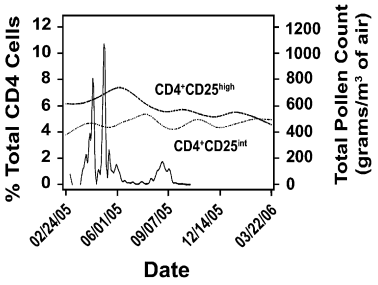

FIG 4. Seasonal changes in $CD4^+CD25^{high}$ and $CD4^+CD25^{int}$ T-cell counts and total pollen counts. T-cell curves were fitted according to each 50 values and pollen curves according to each 10 values. One grain increase per m^3 of air in the 1-month average total pollen count preceding blood draw was associated with 0.10% (95% CI, 0.050, 0.15) increase in % $CD4^+CD25^{high}$/CD4 ($P < .001$).

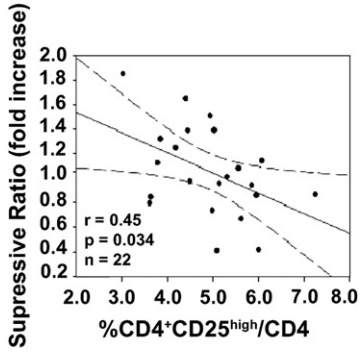

FIG 5. Correlation between % $CD4^+CD25^{high}$/CD4 T cells and suppressive capacity of $CD4^+CD25^+$ T cells. Suppressive ratio was defined as $CD4^+CD25^+$ depleted/1:4 $CD4^+CD25^+$ enriched PBMCs; larger indices represent greater $CD4^+CD25^+$ suppressive capacity.

other ($P = .56$), active atopic dermatitis ($P = .85$), or active wheezing ($P = .88$) in univariate analyses (data not shown).

Finally, we compared CD4CD25 T-cell phenotype with the capacity of PBMCs to secrete selected cytokines in response to phytohemagglutinin stimulation. The proportion of $CD4^+$ T cells expressing intermediate amounts of CD25 correlated positively with IL-5 ($r = 0.35$; $P = .0010$), IL-10 ($r = 0.29$; $P = .0071$), and IL-13 ($r = 0.42$; $P < .0001$; Fig 6, *A-C*; see this article's Table E2 in the Online Repository at www.jacionline.org) production by phytohemagglutinin-stimulated total PBMCs. There was no correlation between $CD4^+CD25^{int}$ T cells and IFN-γ responses, or between $CD4^+CD25^{high}$ cells and any of the cytokine responses.

DISCUSSION

On the basis of findings of studies involving adults with seasonal allergies, we hypothesized that greater numbers of $CD4^+CD25^{high}$ T cells would be associated with lower rates of allergy and atopic markers. On the contrary, we found a positive relationship between $CD4^+CD25^{high}$ T-cell count and both total and allergen-specific IgE. Furthermore, there was a negative association between the percentage of circulating $CD4^+CD25^{high}$ cells and suppressive capacity in the functional assay. Together, these findings suggest the possibility that the CD4 $CD25^{high}$ subset contains activated as well as regulatory T cells. This theory is further supported by evidence of a spring seasonal peak in $CD4^+CD25^{high}$ T cells that closely follows the peak pollen counts in our area, and by demonstration that even the brightest $CD4^+CD25^+$ cells do not uniformly express FOXP3.

The suppressive capacity of $CD4^+CD25^+$ T cells in our subjects was variable, as has been reported in previous studies.[7,9] It is important to consider that our subjects were all from atopic families. In this light, Bellinghausen et al[6] reported that suppressive function of $CD4^+CD25^+$ cells was defective in atopic subjects with high IL-4 and IL-10 responses. Furthermore, Francis et al[4] reported a positive correlation between the proportion of $CD4^+CD25^+$ T cells and proliferation of PBMCs in atopic individuals. In comparing results of atopic and nonatopic individuals, these studies suggest at least 2 possibilities. First,

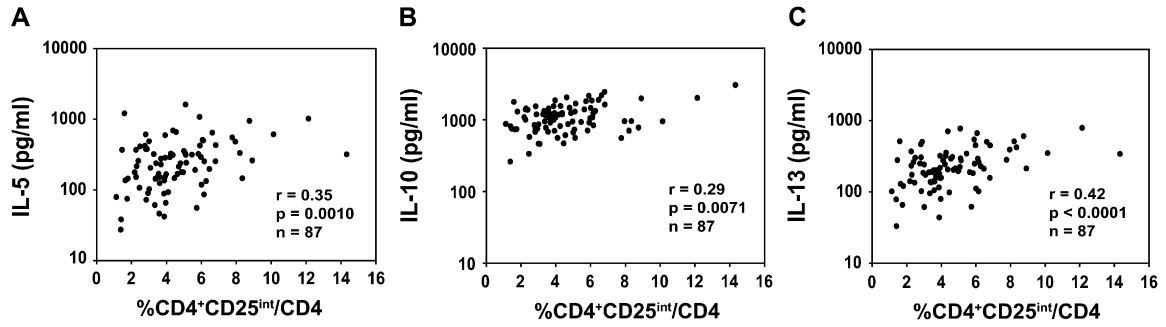

FIG 6. Correlations between % $CD4^+CD25^{int}$/CD4 T cells and IL-5 **(A)**, IL-10 **(B)**, and IL-13 **(C)** production by phytohemagglutinin-stimulated PBMCs.

$CD4^+CD25^+$ T cells from atopic individuals could be functionally impaired. Alternatively, $CD4^+CD25^{high}$ cells from such individuals could represent a mixture of regulatory and activated T cells: proliferative responses of the latter population could thereby mask the effect of regulatory T cells.

In agreement with others,[18] we showed that there is overlap in the CD25 expression of activated versus regulatory T cells, thereby limiting the utility of this marker to distinguish specific T-cell functional subsets. The transcription factor FOXP3 has been shown to be a key regulatory gene for the development and function of regulatory $CD4^+CD25^+$ T cells and is selectively expressed by these cells.[11,12,19] Notably, although FOXP3 expression increased with the level of CD25, even the brightest $CD4^+CD25^+$ cells contained some $FOXP3^-$ cells.

The seasonal variation in our clinical specimens suggests that natural exposures, perhaps related to pollen, induce $CD4^+CD25^{high}$ cells *in vivo*, and these $CD25^{high}$ cells appear to be a mixture of activated and regulatory cells. For example, $CD4^+CD25^{high}$ cells correlated positively with total IgE, and inversely with $CD4^+CD25^+$ suppressive capacity, suggesting the presence of activated cells. On the other hand, there was a negative correlation between % FOXP3/$CD4^+25^{brightest}$ cells and total IgE, suggesting a regulatory function for this cell subset. Interestingly, activation of $CD4^+CD25^-$ cells can induce CD25 expression *in vitro*, and a subset of these activated cells synthesizes FOXP3[19,20] and has suppressive capacity.[19,21] Considering these findings, we speculate that the number of T_{reg} cells may increase as a consequence of allergen exposure and subsequent T-cell activation. Furthermore, the association between pollen counts and % $CD4^+CD25^{high}$ cells was stronger in children with allergen-specific IgE, although this was not specific for pollen. It is conceivable that variations in T_{reg} cell numbers could also be related to seasonal alterations in other allergens. For example, house dust mite levels vary seasonally in the midwestern United States and generally peak in the summer.[22] In addition, there may be other immunologically important seasonal allergens that were not in our testing panel. Finally, the seasonal variation in T_{reg} cells may have also been driven by nonallergenic factors, such as exposure to seasonal infectious diseases. Additional studies are needed to confirm whether the pattern of response is truly unique in allergic versus nonallergic individuals.

The $CD4^+CD25^{int}$ T cells in our study probably represent mainly activated T cells, because the percentage of these cells, but not $CD4^+CD25^{high}$ T cells, was associated with pollen sensitization in spring and strongly with T_H2 cytokine (IL-5 and IL-13), but not IFN-γ, responses.

Previous studies to relate the number or function of peripheral blood $CD4^+CD25^+$ T cells to other atopic phenotypes such as wheezing and atopic dermatitis have shown partly conflicting results. In subjects with atopic dermatitis, a normal or increased number of $CD4^+CD25^+$ T cells with normal immunosuppressive activity has been reported.[23,24] In patients with asthma, increased $CD4^+CD25^+$ T cells have been reported during acute exacerbation.[25,26] We found no differences in T_{reg} function related to atopy, active atopic dermatitis, or a history of wheezing illnesses, although the number of observations was small (n = 22). Neither were there differences in the percentages of $CD4^+CD25^+$ subsets (n = 151). However, boys had higher $CD4^+CD25^{high}$ T-cell counts than girls by univariate analysis, and this suggests that there may be sex differences in development of T_{reg} cells to correspond with sex-related differences in immunologic responses and the prevalence of atopic diseases.[27]

Our study has some limitations. The available blood sample volume for the functional assay was rather small in this study involving young children, which limited the types of functional assays that were feasible. Depletion of $CD4^+CD25^+$ T cells may not be an optimal method to assess suppressive function, although many studies have done so.[3,7,10] Although the sample size of our seasonal data is larger than previous studies assessing seasonal effects, additional power is desirable for interaction analysis related to season, pollen sensitivity, and blood cell phenotype. Finally, all the study participants were from allergic families, and additional studies are needed to evaluate these relationships in unselected populations. Although all of the children in the COAST study have atopic family histories, there are many healthy children in the study with no clinical or biological evidence of

allergic diseases, and these serve as our main control group. We believe that the significant differences between atopic and nonatopic children in COAST are noteworthy and significant in understanding links between familial predisposition and the development of atopic diseases. Additional studies are being designed to evaluate these relationships in unselected populations, as well as in children living in urban rather than suburban locations.

In conclusion, despite previously published suggestions that $CD4^{+}CD25^{high}$ T cells have high regulatory activity, our findings in children do not support a straightforward relationship between CD25 phenotype and function. In fact, our findings suggest that $CD4^{+}CD25^{high}$ T cells are seasonally regulated by environmental factors, and are likely to represent a mixture of activated and regulatory T cells, especially in atopic children.

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