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Longitudinal Relationship of Early-Life Immunomodulatory T-cell Phenotype and Function to Development of Allergic Sensitization in an Urban Cohort

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Abstract

Background—Immunomodulatory T-cells are thought to influence development of allergy and asthma, but early-life longitudinal data on their phenotype and function are lacking.

Objectives—As part of the Urban Environment and Childhood Asthma (URECA) study, we investigated the development of immunomodulatory T-cell phenotype and function, and characterized their relation to allergic disease progression from birth through to age two years.

Methods—Immunomodulatory T-cell phenotype and function in cord blood mononuclear cells and peripheral blood mononuclear cells at age 1 and 2 years were characterized by analyzing CD25^{bright} and FoxP3⁺ expression; proliferative responses and cytokine production. The relation of immunomodulatory T-cell characteristics to allergic sensitization and disease at 1- and 2-years was investigated.

Results—The proportion of CD4⁺CD25^{bright} and CD4⁺CD25⁺FoxP3⁺ T-cells (n=114, 83, 82 at birth, 1- and 2-years respectively) increased significantly, while there were no significant changes in the suppressive function of CD25⁺ T-cells (n=78, 71, 81 at birth, 1- and 2-years respectively). Birth immunomodulatory T-cell characteristics were not related to subsequent allergic

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sensitization or disease. However, increases in the numbers of CD4⁺CD25^{bright} cells and their ability to suppress lymphoproliferative responses at 1 year of age were associated with reduced allergic sensitization at ages 1 ($p<0.03$) and 2 ($p<0.02$) years. Production of the anti-inflammatory cytokine IL-10 by CD25⁺ T-cells appeared to mediate this protective suppressive function. In contrast, by two years of age, we observed the emergence of a positive association of CD4⁺CD25⁺FoxP3⁺ T-cell numbers with allergic sensitization ($p=0.05$) and eczema ($p=0.02$).

Conclusions and Clinical Relevance—These findings suggest that the relationship between immunomodulatory T-cell subsets, allergic sensitization, and eczema is developmentally regulated. In the first year of life CD4⁺CD25⁺ IL-10 producing T-cells are associated with a reduced incidence of allergic sensitization. Once allergic sensitization or eczema are established, CD4⁺CD25⁺FoxP3⁺ T-reg cell expand to potentially counteract the allergic inflammatory response. Understanding the relationship between development of immunoregulatory T cells and early-onset atopy could lead to new preventive strategies for allergic diseases.

Keywords

T regulatory cells; asthma; allergy; IL-10; newborn; suppressive index; toddler; CD4⁺CD25⁺FoxP3⁺

Introduction

Epidemiologic evidence strongly suggests that allergy and asthma originate in early childhood, and that early life exposures are likely to affect immune development and the risk of allergy or asthma. In particular, there is great interest in defining the relationship between the development of immunomodulatory T-cells and the onset of allergic diseases[1, 2].

Cells with immunomodulatory or immunosuppressive capacity, often referred to as regulatory T-cells (T-regs), are essential for maintenance of immunological tolerance to self-antigens and are critically involved in controlling inflammatory responses. These T-cells can exert their inhibitory functions on a range of host cells [3, 4] and suppress proliferation and inflammation through a variety of different mechanisms including cell-cell contact inhibition, competition for growth factors, cytotoxicity and the secretion of anti-inflammatory cytokines such as IL-10 and TGF- β [3, 5].

In broad terms there are two major families; naturally-occurring (or thymus derived) T-reg cells that typically express high levels of the IL-2 receptor CD25 (CD4⁺CD25^{bright}FoxP3⁺), and adaptive (or peripheral) T-regs which include FoxP3⁺ cells that develop outside the thymus and share most functional features of natural T-regs cells. These cells typically evolve from naïve CD4⁺ T cell populations following exposure to antigen and cytokines such as TGF- β FoxP3⁺ T-reg cells, found in the periphery, are primarily characterized by their production of the inflammation-inhibitory cytokines IL-10 (Tr1 cells) and TGF- β (Th3 cells) and can express varying levels of CD25[1, 6–9]. Tr1 cells have been shown to be of specific relevance in immune homeostasis in the lung and in controlling asthmatic airway inflammation [1,10], while Th3 cells may be more relevant in the gut [11]. Throughout this manuscript, we will typically use the term “immunomodulatory T helper (Th)-cells” to

encompass all phenotypes of Th cells that are potentially exerting immunosuppressive effects.

Significant efforts have been made to identify the role played by regulatory T-cells and immunomodulatory cytokines in the development of asthma and allergy. CD4⁺CD25⁺FoxP3⁺ T cells are present in both allergic and non-allergic individuals. Amongst allergic individuals, these cells have been found to be either reduced in numbers or dysfunctional, but the nature of the allergy-related changes in phenotype or function differ between studies [12, 13]. Studies also demonstrate a substantial reduction in IL-10 levels in patients with asthma as compared to control healthy subjects [6, 14, 15] while children with atopic dermatitis were shown to have impaired production of IL-10 in response to a broad range of environmental stimuli [16]. In addition, a polymorphism in the IL-10 gene promoter resulting in reduced IL-10 is associated with more severe allergic disease [17, 18].

Historically, the majority of clinical studies demonstrating altered or impaired immunomodulatory T-cell phenotypes in allergic individuals have focused on adults or on children with established asthma. Given the fact that allergic symptoms usually present in early life, studies of immunomodulatory T-cell characteristics in the first years of life are of significant interest [19, 20]. Schaub and colleagues recently reported that Treg responses and IL-10 production in the cord bloods of newborns from atopic mothers are impaired compared to those from non-atopic mothers [21]. We however could not replicate this finding in a U.S. urban cohort [22]. In addition, Smith *et al.* demonstrated an association between altered neonatal CD4⁺CD25⁺CD127^{lo/-} Treg cell suppressive function and allergic disease in a small cohort of children who developed egg allergies [23]. Additional studies are needed to define the early life longitudinal development of immunomodulatory T-cell phenotype and function and their influences on allergic disease outcomes.

In this study, we used a longitudinal urban U.S. birth cohort to characterize immunomodulatory T-cell phenotypes (CD4⁺CD25^{bright} and CD4⁺CD25⁺FoxP3⁺) and function (suppressive capacity and IL-10 production by CD25⁺ cells) from birth through to 2 years of age, and we assessed how the development of these cells impacts upon wheeze and allergic disease outcomes. We hypothesized that increases in immunomodulatory T-cell numbers and suppressive capacity would reduce the subsequent risk of developing allergic disease in early life. Furthermore, extrapolating from the literature [24], we anticipated that the role and type of immunomodulatory T-cells might change once allergic disease was established.

METHODS

Study population

Study subjects included a subset of children from the Boston metropolitan area who participated in the URECA (Urban Environment and Childhood Asthma) study. The study population, inclusion criteria and recruitment procedures have been previously described [25]. Table I provides a summary of the study population characteristics. This study was approved by the Institutional Review Boards of Boston University and Brigham and Women's Hospital.

Clinical assessments

Recurrent wheeze—Caretakers of the children were asked every 3 months whether the child had experienced any wheezing. The occurrence and frequency of wheezing was assessed using the Respiratory and Allergy Symptoms questionnaire, and from physical examinations at annual study visits [25]

Allergic sensitization—Blood was drawn at annual study visits to measure total IgE antibodies and allergen specific IgE antibodies to peanut, egg and milk allergens by fluoroenzyme immunoassay (UniCAP, Pharmacia & Upjohn, Diagnostics, Uppsala, Sweden). Due to the low number of positives, analysis of sensitization to cockroach antigen and a series of other antigens tested (Der p, Der f, Cat, Dog, Mouse, Alternaria) were not included in this study. The results are in kUA/L. A specific IgE result ≥ 0.35 kUA/L represents a positive test. This approach identifies sensitization to specific foods, which is a marker of atopy but does not specifically identify food allergy.

Other signs of allergic disease—At annual study visits, complete blood count was performed to calculate the percentage of eosinophils in the total white blood cells. In addition, atopic dermatitis was assessed by questionnaire and an Eczema Area and Severity Index (EASI) form completed at the annual physical examination and at the 3 month home visit [25, 26]. We also report eczema diagnosed by parental or physician report (“any eczema”), but for all analysis, limit the eczema diagnosis only to infants with an EASI score greater than or equal to 1 at either the 3 or 12 month study visits.

Cord and Peripheral Blood Mononuclear Cell (MNC) Isolation

Umbilical cord blood samples were collected by needle/syringe from the umbilical vein after delivery into heparinized tubes. Peripheral venous blood was obtained from the children at scheduled annual study visits. All bloods were processed within 24 hours. Cord and peripheral blood mononuclear cells (MNCs) were isolated by density gradient centrifugation with Ficoll-Hypaque Plus (Amersham Biosciences, UK) as previously described [25].

Proliferation assay for CD25⁺-depleted versus un-depleted MNC

The suppressive capacity of CD25⁺ cells was determined by comparing the lymphoproliferative response of MNC's in response to mitogen stimulation in the presence and absence of CD25⁺ cells as described [22]. The proportions of CD4⁺CD25⁺ cells remaining in the “CD25-depleted” sample was reduced to less than 1% following this protocol.

We were interested in the ability of immunosuppressive T-cells to control a global T-cell activation signal as opposed to just suppression of antigen specific T-cells which most likely would have occurred at very low frequencies. Undepleted or CD25⁺ depleted MNCs (1×10^5 /well) were cultured alone or in the presence of the T-cell mitogen PHA (5 μ g/ml). After 4 days, supernatants were collected for cytokines analyses. T-cell proliferation was assessed by thymidine incorporation [22]. The proliferation index (PI) of each sample was calculated as the ratio of mean counts per minute (cpm) of PHA stimulated cells over mean cpm of

unstimulated cells. Suppressive capacity of the CD25⁺ cells was expressed as suppressive Index (SI), which is a ratio of the PI of CD25⁺-depleted cells over PI of undepleted cells.

Cytokine analysis

Interleukin-10 (IL-10), Interferon- γ (IFN- γ) and Interleukin-2 (IL-2) levels in the cell culture supernatant of undepleted- and CD25-depleted MNC on day 4 following PHA stimulation were quantified using a bead-based multiplex assay (Milliplex, Millipore, Billerica, MA). Before running samples on the multiplex a proportion of supernatant was removed for TGF- β analysis. TGF- β levels in the cell culture supernatant were quantified by ELISA using an R&D Systems DuoSet (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions which involved an initial acid activation followed by neutralization step to activate latent TGF- β to the immunoreactive form.

Flow cytometric analysis

The proportions of CD4⁺CD25^{bright} and CD4⁺CD25⁺Fox3⁺ T-cells as a percentage of the total CD3⁺ T-cell population, present in the cord or peripheral blood MNCs were assessed using the FACSCanto cytometer (BD Bioscience, San Jose, CA) running DiVA acquisition software and analyzed in WinList (Verity Software House, Topsham, ME). We have previously published a detailed description of how each population was defined [22]. The CD4⁺CD25⁺ region was defined based on comparison to an isotype control, the CD25^{bright} population was defined in a two step process, first as the population that was brighter than the CD4⁻CD25⁺ population and next by their slightly dimmer CD4⁺ intensity as originally defined by Baecher-Allan, C. *et al* [27].

Modulation of lymphoproliferative response by recombinant IL-10

Undepleted MNC were cultured as described above in the presence or absence of recombinant IL-10 (100 μ g/ml) (R&D Systems, Inc., Minneapolis, MN). Proliferation was assessed on day 4 by measuring thymidine incorporation. Results are expressed as the mean PI in the presence or absence of IL-10.

Statistical Analyses

The distributions of cytokine levels were skewed; therefore, these parameters were log-transformed to better approximate a normal distribution. The Kolmogorov-Smirnoff test was used to assess the difference in distribution of Treg measures across time points. The Spearman correlation was used to assess associations among Treg parameters, cytokine responses and clinical outcomes. Differences in suppression, CD4⁺CD25⁺Foxp3⁺, and IL-10 production by categorical variables were tested by the Wilcoxon rank-sum test. A p-value of 0.05 indicated statistical significance. For all data represented as box and whisker plots, the median is represented by the horizontal bar within the box. The upper and lower boundaries of the box represent the 25th to 75th percentiles of the data.

Results

Subject characteristics

The baseline characteristics of this high-risk study group have previously been described [22] and a summary of the study population baseline characteristics are shown in Table I. Ninety-five percent of children have a history of parental atopic disease (i.e. asthma, hay fever, or eczema). In follow-up, allergic sensitization and wheeze rates were similar at ages 1 and 2, but rates of eczema fell over time (Table II).

Of the 121 newborns with immunomodulatory studies performed at birth (cord blood), 88 had data at age 1 year, and 86 had data at age 2 years. Of these children, flow cytometric data was available on 114, 83 and 82 individuals respectively, and lymphocyte proliferation data and cytokine analysis available on 78, 71 and 81 individuals respectively. Sample availability at the follow-up visits was limited due to missed visits (12% at 12 months, 16% at 24 months) and inability to collect sufficient blood volume collected from the young study participants (12% at 12 months, 11% at 24 months).

For longitudinal analyses, 52 individuals had flow cytometric data at all three time points; 29 individuals had lymphocyte proliferation data at all three time points, and 26 individuals had cytokine analysis available at all three time points. Clinical characteristics within these subsets were similar to that seen in the entire cohort in our study (Supplementary Table I).

Immunomodulatory T-cells increase in number and functionality over time

The numbers of immunomodulatory T-cells present increased significantly over time. The proportions of CD4⁺CD25^{bright} and CD4⁺CD25⁺FoxP3⁺ cells increased significantly from birth to 1-year. The proportions of CD4⁺CD25^{bright} cells increased again from 1- to 2-years of age (Figure 1). There were no significant changes in the suppressive index (SI) of the CD25⁺ cells, although there was a non-significant trend for increases with age (Figure 1). Similar trends were present for individuals with data available at all three time points. In the subset of children with complete data, the mean proportions of CD4⁺CD25^{bright} (birth= 1.9, 1 year= 2.6, 2-year= 3.0) and CD4⁺CD25⁺FoxP3⁺ (birth= 3.8, 1 year= 4.6, 2-year= 3.9), increased after birth. In addition, in paired analyses the mean SI values at age 2-years were consistently higher than those at birth; however this difference did not reach statistical significance (mean SI at birth 1.1; mean SI at 1-year 1.3; mean SI at 2-years 1.5).

Relation of immunomodulatory T-cell numbers and function with allergic outcomes in the first year of life

Birth immunomodulatory T-cell characteristics were not associated with clinical indicators of allergic sensitization and disease at either 1- (Supplementary Figure 1A) or 2- (Supplementary Figure 1B) years of age. At age 1 year, however, CD25⁺ T-cell suppressive capacity was negatively associated with any allergic sensitization ($p=0.03$) as well as with specific sensitization to peanut ($p=0.03$) and egg (0.001) (Figure 2A). In addition, proportions of CD4⁺CD25^{bright} cells were negatively associated with allergic sensitization to milk protein ($p=0.01$) (Figure 2A) at age 1 year. There were no associations between the numbers of CD4⁺CD25⁺FoxP3⁺ T-cells and any clinical outcomes at this time point.

Relation of immunomodulatory T-cell suppressive function and numbers at age 1 year with allergic outcomes at age 2 years

Suppressive capacity of CD25⁺ T-cells at age 1 year remained negatively associated with any allergic sensitization ($p=0.001$) and with sensitization to milk ($p=0.02$) and egg ($p=0.01$) at age 2 years of age (Figure 2B). Proportions of CD4⁺CD25^{bright} T-cells at age 1 year were negatively associated with the percentage of eosinophils ($p=0.04$) in peripheral blood, but not with allergic sensitization at age 2 years (Figure 2B). Again, we observed no association between the proportion of CD4⁺CD25⁺FoxP3⁺ T-cells at age 1 year and any allergic outcomes at age 2 years. To look at this data in a different way, we made comparisons between individuals who were either sensitized or not sensitized to allergens at age 1 year (Figure 3A) or age 2 years (Figure 3B). The presence of allergen-specific IgE, at either age, was associated with a significant reduction in suppressive capacity of their CD25⁺ cells at age 1. Furthermore we demonstrated that the presence of egg specific IgE levels at age 1 (Figure 3C) and age 2 (Figure 3D) years were also associated with a significant reduction in suppressive capacity of their CD25⁺ cells at age 1 (correlation coefficient = -0.33 ($p<0.001$) and -0.36 ($p<0.01$) for IgE levels at age 1 and 2 years respectively).

Relation of immunomodulatory T-cell suppressive function and numbers at age 2 years with allergic sensitization and disease at age 2 years

We found no association of CD25⁺ T-cell suppressive function or the proportions of CD4⁺CD25^{bright} cells at age 2 years with allergic sensitization at age 2 (Figure 4). However, the proportion of CD4⁺CD25⁺FoxP3⁺ cells was positively associated with allergic sensitization (specific IgE antibodies to peanut proteins ($p=0.05$) and milk proteins ($p=0.06$)) and disease (reported visible eczema ($p=0.03$)), (Figure 4). CD4⁺CD25^{bright} cells at age 2 years were also positively associated with reported visible eczema ($p=0.04$) at this age. Interestingly, when we further characterized the phenotype of these immunomodulatory T-cells we observed a trend towards an increased association between “activated” immunomodulatory T-cells based on CD45RO surface marker expression and an increased incidence of allergic sensitization and disease. CD4⁺CD25^{bright}CD45RO⁺ cell numbers were positively associated with recurrent wheeze ($r=0.26$, $p=0.04$), while CD4⁺CD25⁺FoxP3⁺CD45RO⁺ were associated with increased levels of peanut specific IgE ($r=0.27$, $p=0.05$) (Supplementary Figure 2).

Change in immunomodulatory T-cell numbers from birth through to 2 years and allergic sensitization

Using a longitudinal analytical approach, an increase in CD25⁺ T-cell suppressive capacity from birth to 1 year was associated with reduced occurrence of any allergic sensitization as well as with reduced sensitization to specific food allergens (peanut, egg and milk) at ages 1 and 2 years (Supplementary Figure 3). In contrast, increases in CD4⁺CD25⁺FoxP3⁺ (Supplementary Figure 4) cell numbers from age 1- to 2-years were significantly associated with increased rates of recurrent wheeze at age 2, with a similar trend observed for “any wheeze”.

Allergic sensitization in the first year of life can influence specific immunomodulatory T-cell numbers by age 2 years

We investigated the possibility that allergic sensitization in the first year of life could lead to the subsequent expansion of immunomodulatory/regulatory T-cells in this cohort. Infants who had tested positive for allergen-specific IgE at age 1 year had significantly greater numbers of CD4⁺CD25⁺FoxP3⁺ (p=0.05) cells but not CD4⁺CD25^{bright} cells (p=0.91, data not shown) at age 2 years compared to those who were not sensitized (Figure 5A). To further confirm this association total IgE antibody levels present in the serum at age 1 year demonstrated a significant positive association (p<0.01) with CD4⁺CD25⁺FoxP3⁺ cell numbers at age 2 years (Figure 5B). By age 2 years total IgE levels remained positively associated with CD4⁺CD25⁺FoxP3⁺ cell numbers at 2 years (p=0.013, data not shown).

Relation of IL-10 production by CD25⁺ cells with allergic sensitization

We next tested the hypothesis that immunomodulatory T cells could affect allergic sensitization through the production of IL-10. At 1 year, the data is strongly suggestive of an association between allergic sensitization and reduced IL-10 production (p=0.066) by MNC (4 days of PHA stimulation) (Figure 6A). To investigate the cellular source of IL-10 in our cohort, we compared PHA-induced IL-10 production by MNCs to the proportions of lymphocyte subsets in the peripheral blood at age 1 year. IL-10 levels positively correlated with the percentage of CD4⁺CD25^{bright} cells (correlation coefficient: 0.28, 95% CI: 0.05–0.48) (Figure 6B). A similar trend was observed at age 2 years (data not shown).

To further identify CD25⁺ cells as a source of IL-10, we measured PHA-induced IL-10 production on day 4 in undepleted and CD25-depleted MNCs from the children at age 1; CD25 depletion significantly reduced IL-10 production (Figure 6C). A similar effect was seen at age 2 (median levels = 207 vs. 52, for undepleted and CD25-depleted cells respectively, p<0.0001). When we measured IL-2, IFN- γ and TGF- β levels in the culture supernatants we observed no difference in cytokine production between undepleted and CD25-depleted MNCs from the children at age 1 (Supplementary Table II), suggesting that the depletion process is exclusively depleting an IL-10 producing immunomodulatory T-cell type. Finally, we investigated the ability of recombinant IL-10 to suppress PHA induced proliferation using an *in vitro* assay. Un-depleted total mononuclear cells were isolated at 2 years of age and stimulated with PHA for 4 days in the presence or absence of recombinant human IL-10 (100 μ g/ml). Proliferation index was clearly reduced in the presence of rIL-10. Although this reduction did not reach statistical significance (p=0.055) we believe that a 59% reduction in proliferation index (mean PI = 437.7 and 279.2 in untreated and rIL-10 treated MNC) represents a biologically significant result (Figure 6D).

Discussion

In this longitudinal study from birth through age two years, we found evidence that the evolution of immunomodulatory T-cell phenotype is related to the development of allergic sensitization in a time-dependent fashion. Immunomodulatory function at birth was low, and did not predict subsequent atopy. By age 1 year, we found that the suppressive function of CD4⁺CD25⁺ immunomodulatory T-cells (which were likely to be IL-10 producers) was

cross-sectionally and prospectively associated with reduction in allergic sensitization. The initial proportions of CD4⁺CD25⁺FoxP3⁺ cells present were not predictive of subsequent allergy or allergic disease. However, established allergic sensitization or eczema was associated with expanded CD4⁺CD25⁺FoxP3⁺ immunomodulatory T-cells, which appeared to have an activated or memory phenotype, suggesting a possible regulatory response to counteract the inflammatory process. Whether these allergic disease-associated CD4⁺CD25⁺FoxP3⁺ cells are fully functional (suppressive) and successful in downregulating systemic or compartment-specific allergic inflammation is an open question.

From this study we can conclude that the immunomodulatory T-cell types involved in reducing allergy risk may differ from cell types involved in responding to the inflammatory process in established allergy. Our data suggest that in the first year of life, the suppressive immunomodulatory T-cells involved in reducing the risk of allergic sensitization are CD25⁺FoxP3⁻ IL-10 producing cells, which may represent adaptive Tr1 cells. We observed a lack of association of the phenotypic marker FoxP3 with protection against allergic sensitization in the first year of life. In contrast, as allergic disease was established, by age 2 years, the emergence of an association of the FoxP3 marker with the allergic process suggests that a different cell type, CD4⁺CD25⁺FoxP3⁺ predominated or become more functionally responsive. Previous studies have shown that allergic rhinitis and active asthma can result in an expansion of adaptive regulatory T-cells, which generate a regulatory feedback loop to control the inflammatory disease [28, 29] and that CD4⁺CD25^{bright} cells are seasonally increased as a consequence of allergen exposure [30]. While T-reg cells have also been shown to be present in eczematous skin lesions, their ability to suppress the focal allergic process however is uncertain [31].

These CD4⁺CD25⁺FoxP3⁺ cells are likely derived from naive CD4⁺ cells that are expanded upon antigen activation and up-regulate FoxP3 expression upon exposure to TGF- β , which is induced as a protective mechanism during the allergic inflammatory response [32]. In support of this we observed that TGF- β production by total mononuclear cells was significantly elevated in individuals sensitized to peanut allergen at age 2 years (data not shown).

Our data support the hypothesis that the immunomodulatory (perhaps Tr1) T-cells, which protect against allergy development in the first year of life may at least in part be mediating their suppressive effects through production of the anti-inflammatory cytokine IL-10. Previous clinical studies have highlighted the importance of this cytokine's immunosuppressive and anti-inflammatory actions in protecting against the development of asthma and allergic disease [6, 10]. Similar to previous studies, we demonstrated reduced IL-10 production in allergic individuals at age 1 year. We then attempted to further pinpoint the cellular source of IL-10 with an interest in specifying whether IL-10 production by CD25⁺ T-cells was in part mediating their suppressive functions. Our data suggest that CD25⁺ cells are a significant contributor, but are not the sole source of IL-10 production in the MNC of this cohort, consistent with previous studies demonstrating the production of IL-10 by a range of innate and adaptive cell types [33]. In addition, we demonstrated that recombinant IL-10 was capable of suppressing lymphocyte proliferation in this assay system. These findings suggest that the mechanism of suppression observed in our assay

system is through IL-10 production by Tr1 cells as opposed to suppression induced by CD4⁺CD25^{bright}FoxP3⁺ T-reg cells [34, 35]. To establish the precise cellular source of IL-10, additional studies using intra-cellular cytokine staining in conjunction with surface marker staining would be required.

In contrast to immunomodulatory T-cell characteristics at age one, T-cell characteristics at birth (either phenotype or function) had no relation to subsequent allergic sensitization or disease. Our findings are in contrast to a previous study by Smith *et al.* which have shown an association between reduced suppressive function of CD4⁺CD25⁺CD127^{lo} cells at birth with the development of egg allergy in the first year of life, although the discrepancies between our results may simply reflect the differences in specific immunomodulatory T-cell subsets that were analyzed [23]. Our previous studies have shown that neonatal immunomodulatory T-cells possess a naive phenotype (based on surface expression of the activation markers CD45RO, HLA-DR and CD69) and a reduced capacity to suppress lymphoproliferative responses compared to mature T-cells [22]. While suppressive capacity did not reach adult levels, preliminary data demonstrate an increase in activation marker expression over the first 2 years of life suggesting a significant maturation of the immunomodulatory T-cell population. The CD4⁺CD25⁺ T-cell populations acquired more of a memory phenotype over time, based on expression of the surface marker CD45RO (proportions of CD4⁺CD25^{bright}CD45RO⁺ cells at 1 year=1.9±1.2 and at 2 years = 2.7±1.3 mean±SEM). Collectively, these findings suggest that postnatal maturation of immunomodulatory T cells in the first year may play a critical role in determining the risk for allergic sensitization.

Significant controversy exists regarding the characterization of T-cells with immunomodulatory or regulatory function mainly due to the lack of specific cell surface markers for individual cell types. Recently, CD127 (the IL-7 receptor α chain) expression has been used to better discriminate T-reg cells (CD127⁻) from conventional T-cells (CD127⁺) (36). While this marker had yet to be associated with Treg cells at the beginning of the study, cells obtained from the age 2 cohort were assessed for CD127 expression. The proportions of CD4⁺CD25⁺CD127⁻ cells correlated closely with the proportions of CD4⁺CD25⁺FoxP3⁺ cells ($r^2 = 0.87$, $p < 0.001$). We did not observe any significant associations between CD4⁺CD25⁺CD127⁻/^{dim} cell numbers and clinical indicators of allergic sensitization or disease at age 2 years (data not shown). However when we characterized cells as being positive for FoxP3 and negative/dim for CD127 (CD3⁺CD4⁺CD25⁺FoxP3⁺CD127⁻/^{dim}) we did observe a positive association between these cells and total IgE levels ($r = 0.3$, $p < 0.01$) at age 2 years (data not shown). These results suggest that expression of FoxP3 as opposed to a lack of expression of CD127 on CD4⁺CD25⁺ T-cells may represent a more robust marker for the immunomodulatory T-cells that are elevated during allergic sensitization or disease in this cohort.

While postnatal immunomodulatory T-cell function and phenotype were linked to allergy, they did not consistently predict wheeze. This is likely due to the non-allergic etiology of a significant portion of early life wheeze. It is well-established that a significant proportion of infants have transient early wheezing in the setting of viral respiratory infections, because of non-allergic inflammatory obstruction of their small airways [37].

A major strength of this study is the longitudinal study design that involved serial measurements using the same biological assay which, allowed us evaluate time sequence effects during a period of rapid development in Treg phenotype and function and the acquisition of allergic diseases and biomarkers. However, because this longitudinal study involved very young children, we were very limited in the volume of blood that could be collected. The limited numbers of blood cells available therefore precluded the use of standard Treg functional assays in which CD25⁺ cells are isolated, phenotyped, and then added back to effector cell populations. Instead, we compared lymphoproliferative responses in mononuclear cell samples before and after CD25⁺ cell depletion to estimate Treg function, as previously described [24]. One limitation of this assay is that CD25⁺ cells represent a heterogeneous population that includes immunomodulatory T-cells and some effector T-cells. Preliminary experiments however, verified that our protocol preferentially depleted CD25^{hi} cells, which are predominantly regulatory cells. Using this functional assay, we previously observed that the T cell proliferation increased after CD25 depletion for the mothers, but not the newborns in this study [22]. When considered together with the correlations that we observe here between SI in the infants and allergic sensitization, these findings provide evidence that the SI index is a biologically relevant measure of CD25⁺ cell regulatory function. Furthermore, this regulatory activity is immature in infants compared to adults, but shows signs of developmental maturation in the first few years. In addition we demonstrate similar levels of effector cytokine (IL-2 and IFN- γ) production between undepleted MNC and CD25-depleted MNC, which suggests that similar levels of effector T-cells are present in the depleted and undepleted cell populations. A further perceived limitation was that our study was comprised of a high risk U.S., predominantly African American or Hispanic/Latino urban cohort, and these observations will need to be compared to those in other populations. [22]. This however is a strength as well as a limitation, as allergic disease and asthma prevalence and morbidity are high and occur at an early age in this subset of the US population. Thus understanding similarities and differences between immune mechanisms leading to allergy and asthma in this cohort, compared to other cohorts, may ultimately have therapeutic implications.

Distinct T-cell phenotypes with immunomodulatory function may be involved at different stages in the course of development of allergic disease. Our data suggest that in a cohort of at risk urban children, CD25⁺ IL-10 producing cells with suppressive function (potentially Tr1 cells) are protective against allergic sensitization in the first year of life. However, as these children develop allergic disease, their immune systems react to the allergic state and respond with the expansion of (most likely adaptive) CD4⁺CD25⁺FoxP3⁺ T-reg cells. A previous study has also shown that higher numbers of regulatory T-cells (CD4⁺CD25⁺CTLA4⁺) are associated with inhalant sensitization at age 2 years [38]. In our study it appears that allergic inflammation may induce CD4⁺CD25⁺FoxP3⁺ cells, it is not clear however, whether these cells have normal suppressive function in affected individuals. Long-term follow up of these children will be important to establish if the CD4⁺CD25⁺FoxP3⁺ T-cells that are elevated in allergic children at age 2 years will continue to correlate with atopy thereby having the potential to regulate their allergic clinical phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

T-reg	Regulatory T-cell
Th	T-helper cell
IL-10	Interleukin 10
TGF-β	Transforming growth factor β
IgE	Immunoglobulin E
SI	Suppressive Index
PI	Proliferation Index
MNC	mononuclear cells
EASI	Eczema Area and Severity Index

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Figure 1. Immunomodulatory T-cells increase in number and functionality over time

SI of CD25⁺ cells and proportions of CD4⁺CD25^{bright} and CD4⁺CD25⁺FoxP3⁺ cells were assessed over time. Wilcoxon rank sum testing evaluated changes in cellular characteristics between time points. Bars represent median values. P-values indicated on graph for comparisons of cellular characteristics between time points.

Figure 2. Immunomodulatory T-cell numbers and suppressive function, in first year of life are negatively associated with allergic sensitization

Spearman correlations evaluated the relation of SI, and proportions of CD4⁺CD25^{bright} and CD4⁺CD25⁺FoxP3⁺ cells at age-1 to clinical outcomes at 1 (A) and 2 (B) years. Correlation coefficients (circles) and direction of associations are indicated. Significant associations (p<0.05) are highlighted by closed circles. P-values for each association listed on right of each panel.

Figure 3. Suppressive function of CD25⁺ T-cells at age-1 are reduced in allergic individuals at 1- and 2-years

Wilcoxon rank sum test compared suppressive index at age-1 in those that had a positive or negative RAST test at 1-year (A) and 2-years (B) and a positive RAST test to Egg specific IgE at 1-year (C) and 2-years (D).

Figure 4. Immunomodulatory T-cell numbers and suppressive function, at age2 years are not negatively associated with allergic sensitization at age 2 years

Spearman correlations evaluated the relationship between SI, proportions of CD4⁺CD25^{bright}, CD4⁺CD25⁺FoxP3⁺ at 2-years, to clinical outcomes at 2-years. Correlation coefficients (circles) and direction of associations are indicated. Significant associations (p<0.05) are highlighted by closed circles. P-values for each association listed on right of each panel.

Figure 5. Allergic sensitization in the first year of life influences CD4⁺CD25⁺FoxP3⁺ T-cell numbers by age-2

Wilcoxon rank sum test compared proportions of CD4⁺CD25⁺FoxP3⁺ cells at age-2 in individuals that had a positive or negative RAST test at 1-year (A). Spearman correlations evaluated relationship between proportions of CD4⁺CD25⁺FoxP3⁺ cells at age-2 with total IgE levels at 1-year (B). Correlation coefficient and p-value (parenthesis) indicated on graph.

Figure 6. IL-10 production by CD25⁺ T-cells mediates suppressive function and protects against allergic sensitization at age-1

Wilcoxon rank sum test compared PHA-induced IL-10 production by MNC at age-1 in individuals that had a positive or negative RAST test at 1-year (A). Spearman correlation evaluated associations between IL-10 and proportions of CD4⁺CD25^{bright} cells at 1-year. Correlation coefficient and p-value (parenthesis) indicated on graph (B). Wilcoxon rank sum test compared PHA-induced IL-10 production by total MNC and CD25-depleted MNC at 1-year (C). MNC proliferation in response to PHA was assessed at 2-years in the presence or absence of rIL-10 (D).

Table I

Study population characteristics

	N	
Sex of Baby: Male	121	55% (67)
Type of delivery:	121	
Spontaneous vaginal delivery		55.4% (67)
Assisted vaginal delivery		2.5% (3)
Elective (planned) c-section		19% (23)
Non-elective c-section		23.1% (28)
Mother smoked during pregnancy	115	21% (24)
Child was breastfed	110	81% (89)
Duration of breastfeeding (median weeks)	110	9 (1 – 30)
Mother -ever had asthma	114	52% (59)
Father-ever had asthma	103	28% (29)
Mother - hay fevers	113	45% (51)
Father- hay fever	94	29% (27)
Mother ever had eczema	115	32% (37)
Father ever had eczema	103	16% (16)
NICU admission	121	5.8% (7)
Baby's race/ethnicity:	118	
Hispanic		22.9% (27)
Black		55.1% (65)
Mixed Race		19.5% (23)
White/Asian/Other		2.5% (3)
Smoking in home over course of the year	96	57% (55)
Any dog exposure in last year	96	12% (12)
Any cat exposure in last year:	96	34% (33)

Interquartile range is provided in parentheses with medians. Numbers after percents are frequencies. N is the number of non-missing responses.

Table II

Clinical characteristics of entire cohort *

	Birth N=121	Age 1 Year N=88	Age 2 Years N=86	p-value
Male	59% (71)	58% (51)	51% (44)	0.67 ¹
Allergen-specific IgE (any) [†]		34% (30)	30% (23)	0.56 ¹
Peanut IgE [†]		10% (9)	8% (6)	0.59 ¹
Egg IgE [†]		20% (18)	19% (15)	0.88 ¹
Milk IgE [†]		23% (20)	22% (17)	0.92 ¹
Total IgE (kUA/L)		14.0 (6.3 – 36.5)	21.9 (8.4 – 64.0)	0.13 ²
Any wheeze		52% (46)	48% (41)	0.54 ¹
Wheeze >1 episode		30% (26)	27% (23)	0.68 ¹
EASI score 1		51% (45)	22% (19)	<0.001 ¹
Report of visible eczema (Dr. or parent)		81% (71)	41% (35)	<0.001 ¹
Eosinophils (% of WBC)		2.6 ± 2.0	2.8 ± 1.6	0.087 ²

* Plus-minus values are means ± SD. Interquartile range is provided in parentheses with medians. Numbers after percents are frequencies. N is the number of non-missing responses.

[†] 0.35 kUA/L

Tests used:

¹ Pearson test,

² Wilcoxon test