# Ambient air pollution impairs regulatory T-cell function in asthma

**Kari Nadeau, MD, PhD,[a] Cameron McDonald-Hyman, BA,[a] Elizabeth M. Noth, PhD,[b] Boriana Pratt, MA,[b] S. Katharine Hammond, PhD,[b] John Balmes, MD,[b] and Ira Tager, MD, MPH[b]** *Stanford and Berkeley, Calif*

**Background: Asthma is the most frequent chronic disease in children, and children are at high risk for adverse health consequences associated with ambient air pollution (AAP) exposure. Regulatory T (Treg) cells are suppressors of immune responses involved in asthma pathogenesis. Treg-cell impairment is associated with increased DNA methylation of Forkhead box transcription factor 3 (Foxp3), a key transcription factor in Treg-cell activity. Because AAP exposure can induce epigenetic changes, we hypothesized that Treg-cell function would be impaired by AAP, allowing amplification of an inflammatory response.**

**Objectives: To assess whether exposure to AAP led to hypermethylation of the Foxp3 gene, causing impaired Treg-cell suppression and worsened asthma symptom scores.**

**Methods: Children with and without asthma from Fresno, Calif (high pollution, Fresno Asthma Group [FA], n = 71, and Fresno Non Asthmatic Group, n = 30, respectively), and from Stanford, Calif (low pollution, Stanford Asthma Group, n = 40, and Stanford Non Asthmatic Group, n = 40), were enrolled in a cross-sectional study. Peripheral blood Treg cells were used in functional and epigenetic studies. Asthma outcomes were assessed by Global Initiative in Asthma score.**

**Results: Fresno Asthma Group Treg-cell suppression was impaired and FA Treg-cell chemotaxis were reduced compared with other groups ($P \leq .05$). Treg-cell dysfunction was associated with more pronounced decreases in asthma Global Initiative in Asthma score in FA versus the Stanford Asthma Group. Foxp3 was decreased in FA compared with the Fresno Non Asthmatic Group ($P \leq .05$). FA also contained significantly higher levels of methylation at the Foxp3 locus ($P \leq .05$).**

**Conclusion: Increased exposure to AAP is associated with hypermethylation of the Foxp3 locus, impairing Treg-cell function and increasing asthma morbidity. AAP could play a role in mediating epigenetic changes in Treg cells, which may worsen asthma by an immune mechanism. (J Allergy Clin Immunol 2010;126:845-52.)**

***Key words:*** *Ambient air pollution, asthma, immune system, regulatory T cell, Treg, epigenetics*

Asthma, which is characterized by reversible airway obstruction and inflammation, is the most frequent chronic disease in children. Children with asthma have an increased risk of exacerbations when exposed to higher concentrations of ambient air pollutants,[1-3] and some recent studies suggest that ambient air pollutants, especially those from traffic sources, may increase the risk for new onset of asthma.[4,5] Many children with asthma are atopic—that is, they are allergically sensitized to aeroallergens—on the basis of symptoms and/or results of allergen challenge or detection of allergen-specific IgE.[6,7] Ambient air pollutants have been shown to enhance allergic responses through IgE-mediated pathways. *In vitro* studies by Diaz-Sanchez and colleagues[8-12] have shown that diesel exhaust particles (DEP) enhance IgE production that is mediated through increases in isotype-specific IgE mRNA. These responses can be reproduced with phenanthrene, a polycyclic aromatic hydrocarbon (PAH).[13] In mice, increases in IgE after exposure to DEP are associated with increased IL-4, a signature cytokine for $CD4^+$ $T_H2$-type responses that promote allergies and asthma, in part by increasing allergen-specific IgE production.[14] In addition, controlled nasal exposure of atopic human beings to DEP enhances responses to allergens.[15]

There is growing evidence that regulatory T (Treg) cells play an essential role in inhibiting the proximal pathways of allergic sensitization and IgE production in response to allergen exposure.[16] Treg numbers are reduced in the bronchoalveolar lavage fluid of subjects with asthma,[7,17] and this is accompanied by reduced circulating numbers of Treg cells[18] and impaired chemotaxis of Treg cells to lung epithelial cells.[19] Consequently, impaired function and decreased presence of Treg cells in the lung can lead to worsening asthma pathology.

Forkhead box transcription factor 3 (Foxp3) is important in Treg-cell development and function. Methylation of cytosine-phosphate guanine residues in transcriptional regulatory regions of the Foxp3 gene represses the Foxp3 expression and ultimately Treg-cell function.[20] In contrast, complete demethylation of these transcriptional regulatory regions is associated with and appears to be required for stable Foxp3 expression in Treg cells.[20-25] Thus, it is plausible that environmentally induced increases in methylation at the Foxp3 locus could lead to

From [a]the School of Medicine, Stanford University, and [b]the School of Public Health, University of California, Berkeley.

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Reprint requests: Kari Nadeau, MD, PhD, Stanford University, Division of Immunology and Allergy, 269 Campus Drive, CCSR Building, Room 2115, Stanford, CA 94305-5164. E-mail: knadeau@stanford.edu.

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*Abbreviations used*

CARB: California Air Resources Board
$CD4^+$ T cell: Effector T-cell ($CD4^+CD25^{lo}CD127^{hi}$)
DEP: Diesel exhaust particles
FACES: Fresno Asthma Children's Environment Study
FA: Fresno asthma group
FNA: Fresno nonasthmatic group
Foxp3: Forkhead box transcription factor 3
GINA: Global Initiative for Asthma
HBEC: Human bronchial epithelial cells
PAH: Polycyclic aromatic hydrocarbon
$PM_{2.5}$: Particulate matter ≤2.5 μm with aerodynamic diameter
pp: Percent predicted
SA: Stanford asthma group
SNA: Stanford nonasthmatic group
Teff: Effector T
Treg: Regulatory T ($CD4^+CD25^{hi}CD127^{lo}$)

lower levels of Foxp3 expression and to decreases in Treg-cell function.

Epigenetic modulation by ambient air pollution may play a role in regulating the expression of genes important in the pathogenesis of inflammation in asthma.[26,27] Ambient air pollutants can affect DNA methylation and result in changes in chromatin structure.[28] Air pollutants also have been shown to initiate transformation of $T_H1$ to $T_H2$ cells,[26,27] leading to the proatopic cascade of $T_H2$ cytokines (eg, IL-4, IL-13) found in the bronchoalveolar lavage, sputum, and blood of patients with asthma.[26-28] Recent work has shown that transplacental exposure to airborne PAHs increases DNA methylation of 5' CpG islands of several genes.[13] Liu et al[14] demonstrated that altered methylation in genomic DNA correlated with changes in IgE levels. Baccarelli et al[27] reported changes in the status of overall DNA methylation among adults in association with exposure to black carbon, often an indicator of DEP from traffic sources.

Fresno is located at the southern end of California's Central Valley and is one of the fastest-growing areas of California.[29,30] During the years 2005 to 2007, the population of Fresno was exposed to annual average particulate matter ≤2.5 μm ($PM_{2.5}$) concentrations that exceeded the federal annual standard by over 40%.[1,29,31] The occurrence of asthma is also very high in Fresno. The 2005 lifetime prevalence of asthma in children 5 to 17 years in Fresno County was 34% (95% CI, 25-44) compared with 18% (95% CI, 17-19) for the state of California.[1,29]

Given these observations, we hypothesized that ambient air pollution exposure might worsen asthma in children by inducing epigenetic changes that would impair Treg-cell number and/or function. To test this, we examined the effect of ambient air pollutants on Treg-cell function in children with and without asthma and determined whether there were associations between methylation of relevant genes and any associations with health outcomes. Specifically, we hypothesized that (1) exposure to ambient air pollutants leads to impairment of Treg-cell function; (2) attenuation of Treg-cell function is associated with decreased lung function and increased asthma severity classification in children with asthma; and (3) hypermethylation of the Foxp3 gene, a gene important in Treg-cell development and maturation,[20] is associated with decreased gene product and suppression of Treg-cell function.

## METHODS

All methods and procedures were approved by the University of California, Berkeley, and Stanford University Committees for the Protection of Human Subjects.

### Subjects

We studied 4 groups. Children with and without asthma from Fresno, Calif (high pollution; FA, n = 71, and FNA, n = 30, respectively) and from Stanford, Calif (low pollution; SA, n = 40, and SNA, n = 40) were enrolled as a cross-sectional study. The first group of subjects (FA) consisted of children with asthma who have been followed for up to 8 years as part of the Fresno Asthmatic Children's Environment Study (FACES, R01 HL081521). Subjects were children whose residence was within 20 km of the California Air Resources Board (CARB) compliance monitoring site in Fresno. At baseline, subjects answered a detailed respiratory health and general history questionnaire and underwent prebronchodilator and postbronchodilator spirometry and skin prick testing with 14 aeroallergens antigens common in the Fresno area. The second group consisted of 30 additional subjects from Fresno without asthma (FNA). A third and a fourth group of children were recruited from the Stanford Lucile Packard Children's Hospital as follows: 40 children with asthma based on Global Initiative for Asthma (GINA) guidelines (http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.pdf; SA, third group) and 40 children without asthma (SNA, fourth group). The SA and SNA subjects had resided in the immediate vicinity of Palo Alto, Calif, for at least 8 years, and all their residences were >800 m from a major highway, which minimized exposure to traffic-related pollution.

Subjects from the second through fourth groups were frequency-matched by age and sex to the FA subjects. Subjects were excluded if they had been taking oral immunosuppressives within 5 days of the blood draw, had a chronic disease, and/or had acute infection. Subjects without asthma were defined by (1) normal pulmonary function tests, (2) lack of historical diagnosis of asthma, (3) total IgE (Immunocap, Phadia, Sweden) of <10 IU/mL, and (4) negative skin test results (for the same 14 aeroallergens tested in FA). For subjects with asthma, we used the updated GINA classification scheme for asthma severity (2007), which includes symptoms and lung function components and recognizes that treatment with medication can modify these components.

### Exposure to air pollutants

Hourly, quality-assured, ambient air quality and meteorologic data collected at the First Street monitoring station in Fresno (for Fresno cohort) and in Redwood City, Calif (for Stanford cohort) were obtained from CARB. Subject-specific estimates of annual average PAH exposures were based on land use regression analyses that used 497 PAH measurements at the homes of a subset of the FACES cohort (n = 83). Details are presented in this article's Methods section in the Online Repository at www.jacionline.org.

### Collection and processing of blood specimens

We obtained up to 25 mL whole blood from each subject, and assays were performed by using PBMCs and purified cell subsets. Treg cells ($CD4^+CD25^{hi}CD127^{lo}$) and effector T (Teff) cells ($CD4^+CD25^{lo}$ or $^{neg}$) were purified by flow cytometry after staining of PBMCs with fluorochrome-conjugated mAbs. Treg and Teff cells were then used in standard $^3H$ thymidine proliferation assays, flow cytometry, quantitative PCR, and chemotaxis assays according to published methods.[18,19] Details are provided in the Online Repository in the Methods section.

### Statistical analysis

Between-group means were compared with nonparametric Kruskal-Wallis ANOVA and pairwise posttest comparisons via the Dunn multiple comparison test (Graph Pad Prism Software 5.0; Prism Software, La Jolla, Calif). Linear regressions were fit with intercepts for comparisons between lung function and Treg-cell function and for the comparison between average exposure to PAHs and number of methylated CpG islands. All other regressions were fit

**TABLE I.** Comparison of area ambient pollutant concentrations between Fresno and Palo Alto, Calif, based on CARB compliance monitoring for 2008

| Pollutant | Location of compliance monitor* | |
|---|---|---|
| | Redwood City | First Street, Fresno |
| PAHs, annual average (ng/m$^3$) | 0.6 | 4.4 |
| $PM_{2.5}$, annual average (μg/m$^3$) | 10.5 | 21.2 |
| $PM_{2.5}$, 24-h maximum (μg/m$^3$) | 36.0 | 93.0 |
| $PM_{10}$, 24-h high (μg/m$^3$) | 41 | 78 |
| $O_3$, highest 8-h average (ppb) | 70 | 132 |
| $O_3$, no. of days > state 1-h standard | 0 | 44 |
| $O_3$, no. of days > state 8-h standard | 0 | 86 |

*$PM_{10}$*, Particulate matter with aerodynamic diameter ≤10 μm.
*Redwood City is the compliance monitor within 4.7 km of the Palo Alto residences of the Stanford cohort. All FA subjects live within a circle with the First Street monitor as its center and a radius of 20 km.

without intercepts. The association between GINA symptom scores and percentage of Foxp3-positive Treg cells was evaluated with ordinal logistic regression and Kruskal-Wallis analysis of variance. All regressions were carried in SAS v9.1 (SAS Institute, Cary, NC), and the fit of the models was checked with residual versus predicted plots.

## RESULTS

### Subjects

Ambient air pollution data confirmed contrasts in pollution concentrations between Stanford and Fresno, CA (Table I). By design, all 4 groups (FA, SA, FNA, SNA) were similar in age distribution (Table II). The FNA group had a greater percentage of reported smokers at home (17%) than FA, SA, or SNA (all 10%). Overall, GINA scores were lower in the SA versus FA group, with 77.5% of SA and 32.4% of FA subjects having a score of 1 (Table II). Further demographics and health outcome data for each subject (FA and SA) can be found in this article's Tables E1 and E2 in the Online Repository at www.jacionline.org.

### Treg-cell functional studies most impaired in FA

Treg-cell–mediated suppression of autologous conventional CD4$^+$ T-cell proliferation was impaired in FA subjects compared with SA subjects (Fig 1). Treg cells from SNA showed no impairment of suppressive function of autologous conventional CD4$^+$ T cells, in contrast with FNA. Of all 4 groups, defective Treg-cell function was greatest in FA. Atopic status of a subject did not significantly affect the outcome of the Treg-cell functional assay (data not shown). We tested whether conventional CD4$^+$ T cells overproliferate in the absence of Treg cells. The data demonstrated no change in proliferation among conventional CD4$^+$ T cells in any of the 4 groups (see this article's Fig E1 in the Online Repository at www.jacionline.org). There was no increase in cell death in T cells (data not shown).

The degree of Treg-cell dysfunction was associated with decrements in percent predicted (pp) $FEV_1$ for FA and SA (Fig 2). The slopes of the regressions were similar for SA subjects (N = 40) compared with FA subjects (N = 71; ie, for each unit change in Treg-cell suppression, the absolute amount of change was the same). However, the degree of Treg-cell functional impairment was less in the SA subjects (percent predicted range, ~70% to 95% compared with ~25% to 80% for FA subjects).

Because 1 parameter of Treg-cell function (ie, suppression assay, Fig 1) was attenuated in FA compared to SA subjects, we assessed whether a second parameter of Treg-cell function (ie, chemotaxis assay) would be impaired. We determined whether migration by Treg cells to primary human bronchial epithelial cells (HBECs; Fig 3) was dependent on a specific chemokine pathway. Treg cells from all 4 groups were incubated with blocking anti-CCR4, anti-CCR8, or anti-CCR7 mAbs or, as a negative control, isotype-matched mAbs of irrelevant specificity. Treg-cell chemotaxis was reduced by approximately 2-fold in FA subjects relative to SA subjects (Fig 3). In addition, migration of Treg cells to HBECs was reduced in FNA subjects compared with SNA subjects (Fig 3). For all groups, CCR4-dependent and CCR8-dependent pathways appear to be involved (as indicated by further decreased migration in the presence of anti-CCR4 and anti-CCR8 mAbs). We tested CCR7 as an alternative to CCR4 and CCR8 because T cells have been found to depend on this pathway for ingress to inflamed lungs[30]; however, CCR7 neutralization had no significant effect.

### Molecular studies in Treg cells demonstrate lowest Foxp3 expression and highest IL-4/IL-13 levels in FA

Because Foxp3, IL-10, TGF-β, CD25 (IL-2Rα), signal transducer and activator of transcription 5, and CCR8 are implicated in Treg-cell function,[32-34] real-time PCR was performed to assay the levels of these transcripts in Treg cells. Transcripts for Foxp3, TGF-β, and CCR8 were significantly decreased in FA compared with SA Treg cells (see this article's Fig E2 in the Online Repository at www.jacionline.org).

The mean percentage of Foxp3$^+$ Treg cells was decreased in the FA relative to SA and all other groups (Fig 4, *A*; see this article's Fig E3 in the Online Repository at www.jacionline.org). SNA had the highest mean, which was significantly different from that of the FNA subjects ($P < .05$). We observed a significant association between the percentage Foxp3$^+$ Treg cells and the percentage of Treg cell function in FA (Fig 4, *B*) and in SA. We next evaluated trends within FA and SA subjects to see whether there were any associations between the GINA severity score and total percentage of Foxp3$^+$ Treg cells. In both FA and SA groups, increased asthma severity by GINA classification was associated with a decreased percentage of Foxp3$^+$ Treg cells; however, asthma outcomes were worse as determined by GINA score in the FA group (Fig 4, *C,* vs 4, *D*).

Consistent with the impairment of Treg-cell function, CD4$^+$ T cells purified from FA subjects showed greater skewing toward a $T_H2$ phenotype than the other groups, as measured by intracellular flow cytometry of IL-4 and IL-13 in CD4$^+$ T cells (Fig 5)

### Exposure to ambient concentrations of polycyclic aromatic hydrocarbons is associated with epigenetic changes in Foxp3 locus of Treg cells

We analyzed the methylation status of CpG residues of different *cis*-regulatory elements of the Foxp3 locus. We focused on the promoter and intronic regions (see this article's Fig E4 in the Online Repository at www.jacionline.org), because these are both critical for Foxp3 gene expression.[21] Increased methylation of the 8 CpG islands in the promoter region (see this article's Fig E5 in the Online Repository at www.jacionline.org) and of the 13 CpG islands in the intronic region was detected in Treg cells in the order of FA > FNA > SA > SNA groups (Fig 6, *A*). Moreover, we

**TABLE II.** Characteristics of subject groups

| Demographics | FA N = 71 | FNA N = 30 | SA N = 40 | SNA N = 40 |
|---|---|---|---|---|
| Median age at blood draw (y) (25th, 75th percentile) | 14 (12, 16) | 13 (10, 17) | 12.0 (9, 16) | 12.5 (9, 15.5) |
| Sex (% female) | 42.5 | 50 | 52.5 | 47.5 |
| GINA* | 1 = 32.4% | | 1 = 77.5% | |
| | 2 = 50.7% | | 2 = 12.5% | |
| | 3 = 12.7% | | 3 = 10.0% | |
| | 4 = 4.2% | | 4 = 0% | |
| Positive skin prick text (% yes) | 50.0 (N = 60)‡ | | 75.0 | |
| Mother with asthma (% yes) | 38.2 | | 50.0 | |
| Hospitalized for asthma (% yes) | 14.1 | | 42.5 | |
| ED visit for asthma (% yes) | 2.8 | | 42.5 | |
| Smoker lives at home (% yes)† | 9.7 | 16.7 | 10.0 | 10.0 |
| Median birth weight (kg) (25th, 75th) | 3.4 (3.1, 3.7) (N = 58) | | 3.2 (2.9, 3.7) (N = 36) | |
| Median age at first asthma diagnosis (y) (25th, 75th) | 5 (3, 6) | | 8.0 (7, 9) | |
| Median $FEV_1$% predicted (25th, 75th) | 91.2 (79.9, 100.2) | | 93.0 (85.8, 101) | |

*ED,* Emergency department.
*All characteristics for FA subjects based on baseline data. Baseline evaluation occurred from November 2002 through September 2005, and FA subjects were 6 to 11 years of age at baseline.
†Differences between groups, $P = .76$.
‡Skin prick testing performed only on those without history of previous severe reaction.

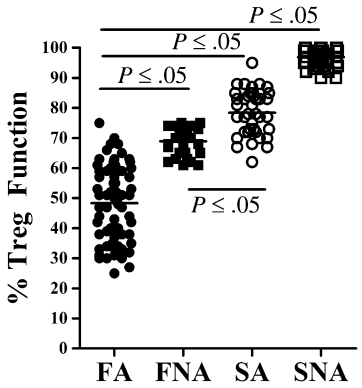


**FIG 1.** Results are the mean of duplicate experiments for each sample. **A,** Treg-cell suppression assay. Suppressive activities of Treg cells among 4 subject groups (FA, n = 71; FNA, n = 30; SA, n = 40; SNA, n = 40). Graphs show degree of suppression at 1:1 ratio of conventional $CD4^+$ T (Teff) cells to Treg (% suppression of proliferation = [(Teff-cell proliferation without Treg cells – Teff-cell proliferation with Treg cells)/(Teff-cell proliferation without Treg cells) × 100]).

found a direct association between average daily PAH exposure in the FA group and the number of methylated CpG islands. Importantly, the exposure estimates were for a period that was an average of 3 years earlier than the blood draw (Fig 6, *B*).

## DISCUSSION

The results from this cross-sectional study provide support for our central hypothesis that ambient air pollution exposure may worsen asthma symptoms, at least in part, by epigenetic decrease of Foxp3 expression and impaired Treg-cell–mediated suppression of $T_H2$ responses. Compared with children who live in a relatively low air pollution environment (Palo Alto, Calif), children who live in an environment with high levels of ambient air pollutants (Fresno/Clovis, Calif) have impaired function of their Treg cells (hypothesis 1). Treg-cell dysfunction and Foxp3 downregulation were most pronounced in FA, but even FNA had impaired Treg-cell function. In addition, a significant inverse association between asthma severity score (Fig 4, *C*) and Treg-cell function was found in FA (hypothesis 2). Consistent with our central hypothesis, the FA group showed greater skewing to a $T_H2$ phenotype, assessed by $CD4^+$ T-cell expression of IL-4 and IL-13. Finally, our data demonstrated an association between estimated average daily exposure to PAHs and the number of methylated CpG islands (hypothesis 3) and a parallel decrease in $FEV_1$ in the FA subjects.

Several other observations emerged that are of additional relevance with respect to air pollution exposure. First, FNA compared with SNA showed evidence of decreased Treg-cell function (Figs 1, *A,* and 3). Mainstream and secondhand tobacco smoke are known to contain many of the same constituents present in ambient air pollution,[26,27] and the FNA group had a higher percentage of secondhand smoke exposure than all of the other groups (Table II). Thus, it is plausible that both air pollution and secondhand smoke exposure could have contributed to impaired Treg-cell function in the FNA group. Second, our data suggest that ambient air pollution exposure in patients with asthma (FA) is associated with lower expression of Foxp3 in Treg cells, worse dysfunction of Treg cells, and more severe asthma severity scores compared with SA. Finally, the failure to find evidence of Treg-cell suppression in age-matched and sex-matched SNA subjects by any measure suggests that the immunopathology of asthma and exposure to air pollution likely make separate contributions to altered Treg-cell function.

We employed GINA severity scores for the asthmatic groups (FA and SA) because there is considerable evidence to suggest that lung function should not be used as the sole criterion for classifying asthma severity in children; assessment of symptoms, disability, health care use, and medication use should also be considered.[1,29] There was a time difference between the GINA score calculation (up to 7 years), PAH exposure (up to 3 years), and the subsequent day of blood for SA and FA subjects. This

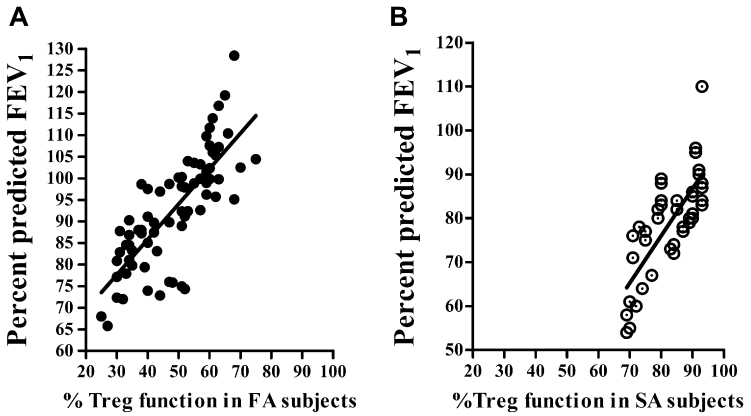


**FIG 2.** Associations between suppression of Treg-cell function and last $FEV_1$ before the blood draw. **A,** Percent predicted $FEV_1$ for FA subjects (n = 71), slope 0.82 (SE = 0.08), $R^2 = 0.60$. **B,** Percent predicted $FEV_1$ for SA subjects (n = 40); slope 1.07 (SE = 0.15), $R^2 = 0.71$. Both regressions fit with intercept.

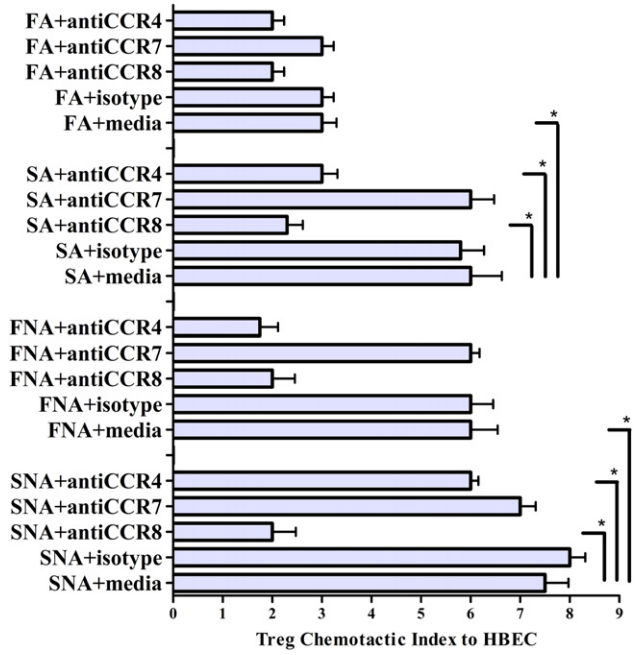


**FIG 3.** Comparison of Treg-cell chemotaxis toward HBEC (human bronchial epithelial cells) in FA (n = 71), SA (n = 40), FNA (n = 30), and SNA (n = 40) on the basis of on Treg-cell chemotactic indices. Each assay was performed in duplicate (*error bars* represent SDs). Blocking antibodies were used against CCR8, CCR7, and CCR4 and compared with controls. **P* value ≤.05 based on Kruskal-Wallis 1-way ANOVA via Dunn multiple comparison test.

could indicate that pollution exposure and the degree of methylation in Foxp3, Treg-cell impairment, and asthma severity score are related to a chronic process rather than an acute process. Interestingly, the SA group had a higher rate of acute asthma exacerbations in association with hospitalization and emergency department visits than the FA group (Table II).

These conclusions need to be placed in the context of several issues related to the study design. The number of subjects was relatively small, which raises questions about the robustness of observations. We also did not adjust the analyses for individual differences that might be associated with differences between subjects with and without asthma within and between Fresno and Stanford, nor did we account for all indoor environmental exposures. However, because subjects were age-matched and sex-matched and did not have any other chronic illnesses, it is unlikely that important confounders were missed. Another observation that lends support to the validity of these results is that the relation between a unit change in percentage of Treg-cell function and pp $FEV_1$ (Fig 2) was similar between FA and SA, and there was virtually no overlap in the actual percentage of suppression between the 2 groups. This indicates that the observed quantitative relation is continuous over the range of pollutant exposure and that the cleaner ambient pollutant environment of Palo Alto relative to Fresno could lead to less attenuation of Treg-cell function, as one would expect in a monotonic exposure-response relationship.

A large body of work has shown that Treg cells suppress other immune cells involved in disease progression in asthma: active T cells, eosinophils, basophils, antigen-presenting cells, and mast cells.[35] We studied $Foxp3^+$ Treg cells because of their association with the control and treatment of allergic asthma. Low Foxp3 expression can lead to Treg-cell dysfunction, and we and others previously have published that asthma severity is associated with impaired peripheral blood Treg-cell function.[7,16-19] Data from this study indicate that exposures to ambient air pollutants appear to contribute independently to this Treg-cell dysfunction, resulting in the enhancement of the $T_H2$-skewed immune state in asthma (Fig 5).

Studies have determined that Treg-cell expression of CCR4 and CCR8 transcripts is under the control of Foxp3 and that these chemokine receptors respond to ligands produced by bronchial epithelium.[36] We found that there is less migration of Treg cells from FA subjects to broncho epithelial cells (BECs) (Fig 3). These results suggest that, in healthy subjects, CCR8 may guide Treg cells to sites of antigen presentation in the lung. However, under conditions of inflammation, such as those that follow exposure to components of ambient air pollution, Treg cells express less CCR8 and thus might not migrate effectively to the lung. This defect, in addition to the impairment in Treg-cell suppression of $CD4^+$ proliferation, could further attenuate the overall function of Treg cells in the lung in patients with asthma.

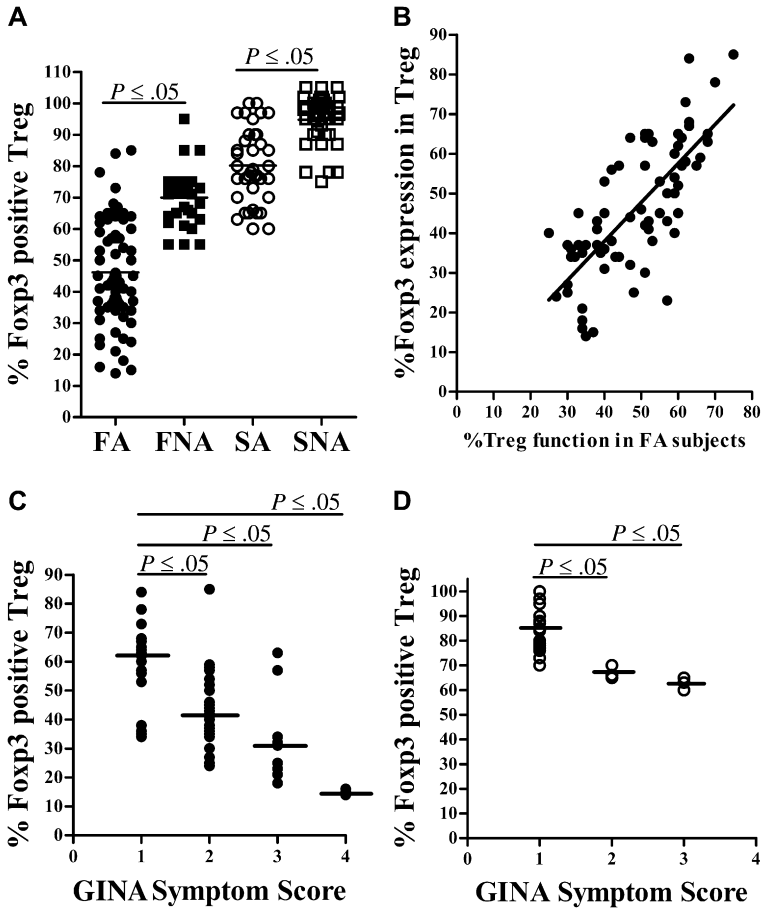


**FIG 4.** **A,** Percentage of Treg cells expressing Foxp3 protein in FA (n = 71), FNA (n = 30), SA (n = 40), and SNA (n = 40). **B,** Association between percentage of Foxp3$^+$ Treg cells and percentage of Treg-cell function for 71 FA subjects. **C,** Percentage of Foxp3$^+$ Treg cells plotted with GINA classification (FA, n = 71). **D,** Percentage of Foxp3$^+$ Treg cells plotted with GINA classification (SA, n = 40). *Horizontal bars* are medians.

Several articles have shown possible mechanisms involved in Treg-cell modulation in human beings through the potential Treg-cell conversion to effector T cells.[20,37,38] Differences in Treg-cell subsets may contribute to our understanding of why asthma/allergy, rather than autoimmune disease, is more affected by ambient air pollution. In addition, we studied the epigenetic modulations of a class of ambient air pollutants, polycyclic aromatic hydrocarbons, on the Foxp3 locus of Treg cells. Our findings are new in that we were able to identify epigenetic changes (ie, CpG methylation patterns) in a subset of immune cells (Treg cells), which could have been overlooked had epigenetic studies occurred in DNA derived from a mixed immune cell population like PBMCs and related this directly to a functional correlate of asthma.

In summary, these findings support our hypotheses that exposure to ambient air pollution is associated with increased methylation of CpG islands in the Foxp3 locus, worsened Treg-cell function, unchecked $T_H2$ polarization, and worsened asthma severity scores. These results also suggest that dose-response relationships may exist for exposure to ambient air pollution and Treg-cell function and/or Foxp3 methylation. Differences were detected in Treg-cell function and phenotypes between subjects exposed to high ambient air pollution levels versus low ambient air pollution levels (Fresno vs Stanford cohorts) and subjects with asthma versus with no asthma. Moreover, our methodology of functional, phenotypic, and epigenetic assays on Treg cells in populations differentially exposed to ambient air pollution could serve as a prototype for a comprehensive evaluation of the effects of pollutant exposures on other immune cell subsets.

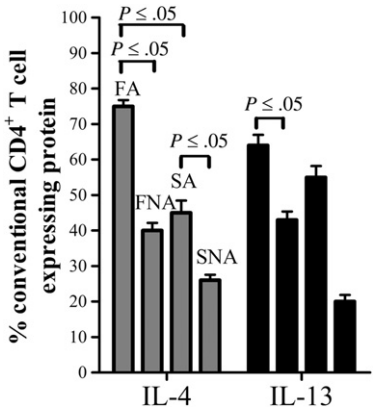


**FIG 5.** Multicolor flow cytometry stained for CD4, CD25, IL-4, and IL-13 (FA, n = 71; FNA, n = 30; SA, n = 40; SNA, n = 40). See previous figure legends for statistical tests and inference.

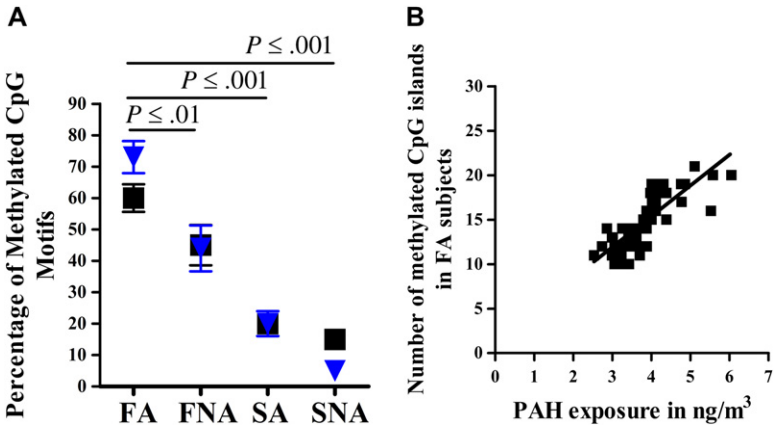


**FIG 6. A,** Percentage of methylated CpG islands from Treg-cell DNA (FA, n = 71; FNA, n = 30; SA, n = 40; and SNA, n = 40). Percentage of methylation in Foxp3 promoter *(black)* out of 8 (*square,* mean), and in Foxp3 intronic *(blue)* out of 13 (*arrow,* mean) CpG islands. **B,** Association between total number of methylated CpG islands in Treg-cell Foxp3 gene and level of PAH exposure (N = 69 FA subjects).

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## REFERENCES

1. Margolis HG, Mann JK, Lurmann FW, Mortimer KM, Balmes JR, Hammond SK, et al. Altered pulmonary function in children with asthma associated with highway traffic near residence. Int J Environ Health Res 2009;19:139-55.
2. D'Amato G. Environmental urban factors (air pollution and allergens) and the rising trends in allergic respiratory diseases. Allergy 2002;57(suppl 72):30-3.
3. Shea KM, Truckner RT, Weber RW, Peden DB. Climate change and allergic disease. J Allergy Clin Immunol 2008;122:443-53; quiz 454-5.
4. Islam T, Gauderman WJ, Berhane K, McConnell R, Avol E, Peters JM, et al. Relationship between air pollution, lung function and asthma in adolescents. Thorax 2007;62:957-63.
5. Jerrett M, Shankardass K, Berhane K, Gauderman WJ, Kunzli N, Avol E, et al. Traffic-related air pollution and asthma onset in children: a prospective cohort study with individual exposure measurement. Environ Health Perspect 2008;116: 1433-8.
6. Bergstrom SE, Boman G, Eriksson L, Formgren H, Foucard T, Horte LG, et al. Asthma mortality among Swedish children and young adults, a 10-year study. Respir Med 2008;102:1335-41.
7. Nguyen KD, Fohner A, Booker JD, Dong C, Krensky AM, Nadeau KC. XCL1 enhances regulatory activities of CD4+ CD25(high) CD127(low/-) T-cells in human allergic asthma. J Immunol 2008;181:5386-95.
8. Diaz-Sanchez D, Jyrala M, Ng D, Nel A, Saxon A. In vivo nasal challenge with diesel exhaust particles enhances expression of the CC chemokines rantes, MIP-1alpha, and MCP-3 in humans. Clin Immunol 2000;97:140-5.
9. Diaz-Sanchez D, Penichet-Garcia M, Saxon A. Diesel exhaust particles directly induce activated masT-cells to degranulate and increase histamine levels and symptom severity. J Allergy Clin Immunol 2000;106:1140-6.
10. Diaz-Sanchez D, Riedl M. Diesel effects on human health: a question of stress? Am J Physiol Lung Cell Mol Physiol 2005;289:L722-3.
11. Diaz-Sanchez D, Tsien A, Casillas A, Dotson AR, Saxon A. Enhanced nasal cytokine production in human beings after in vivo challenge with diesel exhaust particles. J Allergy Clin Immunol 1996;98:114-23.
12. Diaz-Sanchez D, Tsien A, Fleming J, Saxon A. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. J Immunol 1997;158:2406-13.
13. Perera F, Tang WY, Herbstman J, Tang D, Levin L, Miller R, et al. Relation of DNA methylation of 5'-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma. PLoS One 2009; 4:e4488.
14. Liu J, Ballaney M, Al-alem U, Quan C, Jin X, Perera F, et al. Combined inhaled diesel exhaust particles and allergen exposure alter methylation of T helper genes and IgE production in vivo. Toxicol Sci 2008;102:76-81.
15. Mastrangelo G, Veller Fornasa C, Pavanello S, Mercer G, Lazzaro M, Milan G, et al. Polyaromatic hydrocarbons administered in humans by dermal route increase total IgE. Int J Immunopathol Pharmacol 2003;16:145-50.
16. Taylor A, Verhagen J, Akdis CA, Akdis M. T regulatory cells and allergy. Microbes Infect 2005;7:1049-55.
17. Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T-cells in pediatric asthma. J Allergy Clin Immunol 2007;119:1258-66.
18. Nguyen KD, Vanichsarn C, Nadeau KC. Increased cytotoxicity of CD4+ invariant NKT-cells against CD4+CD25hiCD127lo/- regulatory T-cells in allergic asthma. Eur J Immunol 2008;38:2034-45.
19. Nguyen KD, Vanichsarn C, Fohner A, Nadeau KC. Selective deregulation in chemokine signaling pathways of CD4+CD25(hi)CD127(lo)/(-) regulatory T-cells in human allergic asthma. J Allergy Clin Immunol 2009;123:933-9, e910.
20. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? Nat Rev Immunol 2009;9: 83-9.
21. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. Blood 2009;114(18):3727-35.
22. Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, et al. Epigenetic regulation of Foxp3 expression in regulatory T-cells by DNA methylation. J Immunol 2009;182:259-73.
23. Kim HP, Leonard WJ. CREB/ATF-dependent T-cell receptor-induced FoxP3 gene expression: a role for DNA methylation. J Exp Med 2007;204:1543-51.
24. Janson PC, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. FOXP3 promoter demethylation reveals the committed Treg population in humans. PLoS One 2008;3:e1612.
25. Peters JH, Hilbrands LB, Koenen HJ, Joosten I. Ex vivo generation of human alloantigen-specific regulatory T-cells from CD4(pos)CD25(high) T-cells for immunotherapy. PLoS One 2008;3:e2233.
26. Miller RL, Ho SM. Environmental epigenetics and asthma: current concepts and call for studies. Am J Respir Crit Care Med 2008;177:567-73.
27. Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, et al. Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med 2009;179:572-8.
28. Truyen E, Coteur L, Dilissen E, Overbergh L, Dupont LJ, Ceuppens JL, et al. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. Thorax 2006;61:202-8.
29. Balmes JR, Earnest G, Katz PP, Yelin EH, Eisner MD, Chen H, et al. Exposure to traffic: lung function and health status in adults with asthma. J Allergy Clin Immunol 2009;123:626-31.
30. Tager I. Final report on FACES. Berkeley, California: Air Resources Board; 2006: 1-716.
31. Kohlmeier JE, Woodland DL. Memory T-cell recruitment to the lung airways. Curr Opin Immunol 2006;18:357-62.
32. Marson A, Kretschmer K, Frampton GM, Jacobsen ES, Polansky JK, MacIsaac KD, et al. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. Nature 2007;445:931-5.

33. Sakaguchi S, Wing K, Yamaguchi T. Dynamics of peripheral tolerance and immune regulation mediated by Treg. Eur J Immunol 2009;39:2331-6.
34. Cohen AC, Nadeau KC, Tu W, Hwa V, Dionis K, Bezrodnik L, et al. Cutting edge: decreased accumulation and regulatory function of CD4+ CD25(high) T-cells in human STAT5b deficiency. J Immunol 2006;177:2770-4.
35. Umetsu DT. Understanding the immunological basis of asthma; immunotherapy and regulatory T-cells. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M 2006;95):211-4; discussion 215-6.
36. Sekiya T, Yamada H, Yamaguchi M, Yamamoto K, Ishii A, Yoshie O, et al. Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics. Allergy 2002;57:173-7.
37. Bluestone JA, Liu W, Yabu JM, Laszik ZG, Putnam A, Belingheri M, et al. The effect of costimulatory and interleukin 2 receptor blockade on regulatory T-cells in renal transplantation. Am J Transplant 2008;8:2086-96.
38. Bour-Jordan H, Bluestone JA. How suppressor cells led to anergy, costimulation, and beyond. J Immunol 2009;183:4147-9.

# METHODS

## Subjects

Subjects had to report a doctor's diagnosis of asthma with asthma symptoms and an active prescription for asthma medication in the 12 months before the interview. Details of the testing and follow-up of this cohort have been presented. Subjects participated in three 14-day panels each year that included twice-daily spirometry and symptom evaluation, along with a 6-month visit to the field office for an in-depth respiratory history questionnaire plus prebronchodilator and postbronchodilator spirometry. The subjects in this report are a convenience sample selected on the basis of their willingness to provide a blood specimen for the study.

## Exposure assessments

For the FA group, daily pollutant exposures were assigned to subjects on the basis of measurements at the First Street site. Air pollution was measured as the 8-hour daily maximum concentration of ozone ($O_3$) and the 24-hour average concentrations of the following pollutants: $PM_{2.5}$, particulate matter from 2.5 μm to 10 μm in aerodynamic diameter, elemental carbon, PAHs, phenanthrene, $NO_2$, and aerosol nitrate ($NO_3$). $PM_{10\text{-}2.5}$ mass was determined by the difference between particulate matter with aerodynamic diameter ≤10 μm and $PM_{2.5}$ mass measurements using Met One Instruments Beta-Attenuation Mass Monitors (Met One Company, Grant Pass, Ore) (http://www.metone.com). Black carbon (BC) was determined from Aethalometer (Magee Scientific model AE42) measurements of the optical absorption of $PM_{2.5}$ ambient aerosol at 880 nm. Elemental carbon concentrations were estimated from BC measurements (elemental carbon = 1.19*BC). $PM_{2.5}$ nitrate was determined from Rupprecht and Patashnick 8400 Continuous Nitrate Analyzer measurements adjusted for equivalency with collocated filter-based $PM_{2.5}$ nitrate measurements obtained with Harvard Impactors and backup filters ($NO_3$= 1.45*R&P$NO_3$). The daily First Street data were used directly for all pollutants except the PAHs and phenanthrene.

For PAH measurements, based on our previous published methods,[E1] we developed land use regression models for outdoor, daily exposure to phenanthrene and the sum of PAHs with 4, 5, or 6 rings (PAH456) with mixed modeling regression to incorporate both temporal and spatial covariates for FA. From February 2002 to February 2003, intensive PAH sample collection onto quartz filters coated with ground XAD resin (Amberlite, Supelco, Bellefonte, Pa) was conducted at the homes of 83 FACES subjects for a total of 497 outdoor PAH samples (5-10 samples at each home). These samples were analyzed by gas chromatography/mass spectroscopy for individual concentrations of ambient PAHs. In addition, PAH concentration data were collected daily by the PAS2000 monitor at the First Street station in Fresno from November 2000 through February 2007. The PAS2000 provides real-time measurements of total particle-bound PAH. The filter concentrations at subjects' homes were the dependent variable for phenanthrene and PAH456; the candidate covariates in the model included the particle-bound PAH concentrations at the First Street site, meteorologic data, source data (traffic and land use), and other temporal and spatial variables (agricultural burning, season, and so forth). The model developed was combined with the daily meteorologic and PAH data from the First Street site and the individual data for each subject's home to generate estimates of each subject's exposure each day through February 2007, and from these, each subject's individual 2006 annual average PAH exposure, which was used as the metric of PAH exposure for the current study.

**Subjects from Palo Alto.** To provide a comparison for estimated exposure to ambient pollutants for subjects from Palo Alto, we used data from the nearest CARB compliance monitor in Redwood City (a maximum of 4.7 km from the residences in the Stanford cohorts).

## Collection and processing of blood specimens

We obtained up to 25 mL whole blood from each subject. Whole blood specimens were shipped from Fresno to a processing laboratory or were obtained from the Stanford clinics within 12 hours of collection. A shipping control blood sample was shipped and tested to confirm that no shipment artifacts had occurred.

We obtained a complete blood count with differential for each subject. $CD4^+$ T cells were enriched by incubation with the negative selection $CD4^+$ isolation kit (StemCell Technologies, Vancouver, British Columbia, Canada); then, 1:1 heparinized whole blood PBS with 1% BSA at 450*g* was centrifuged for 40 minutes over Ficoll-Hypaque density gradients. The $CD4^+$ T-cell–containing PBMC layer was collected and washed twice with 10 mL PBS at 4°C. After centrifugation at 250*g* for 15 minutes, cells were resuspended in PBS in flow-cytometry staining tubes. Live/Dead staining (Molecular Probes/Invitrogen) was used in all samples. Live cells are identified by the intracellular conversion of a calcein ester to free calcein (intensely fluorescent in the green spectrum), and dead cells are identified by the red staining of internal nucleic acids by ethidium homodimer. Subsequently, the live cell fraction was stained with CD4–fluorescein isothiocyanate (clone SK3; BD Biosciences, San Jose, Calif), CD25–phycoerythrin (clone 4E3; Miltenyi Biotec, Auburn, Calif) and CD127 (IL-7 receptor α-chain) allophycocyanin (clone SB199; BioLegend, San Diego, Calif) antibodies and sorted by flow cytometry for live $CD4^+CD25^{hi}CD127^{lo/-}$ Treg cells (FACS Aria; BD Biosciences). CD127 depletion occurred to ensure separation of activated conventional T cells from stable Treg-cell populations, which allows for the isolation of highly enriched $Foxp3^+$ cells.[E2-E5] Live non-Treg ($CD4^+CD25^-$) cells (subsequently called conventional effector $CD4^+$ T cells) were flow-sorted simultaneously. Multicolor flow-cytometry staining for $CD4^+CD25^{hi}CD127^{lo}$ (Treg) cells and $CD4^+CD25^-$ (conventional $CD4^+$ T) cells demonstrated that each cell population was routinely >95% pure. The Treg and conventional $CD4^+$ T cells were incubated in RPMI-1640 media, 10% FBS, and 1% L-glutamine after purification for 2 hours before undergoing further experiments.

## Treg-cell suppression assays

Assays were performed in round-bottom 96-well microtiter plates according to previously published methods[E2-E5] with fixed numbers of live cells per well for each cell type: $CD4^+$ T cells, Treg cells, and antigen-presenting cells (APCs). In brief, autologous $CD4^+CD25^-$ conventional T cells, and autologous-irradiated CD3-depleted PBMCs (APCs) with or without autologous Treg cells, were added. APCs were depleted of T cells by using StemSep human $CD3^+$ T-cell depletion (StemCell Technologies), followed by irradiation with 40 Gy. All wells were supplemented with anti-CD3 mAb (clone HIT3a at 5.0 μg/mL). T cells were cultured for 7 days at 37°C in a 5% $CO_2$-humidified atmosphere. Sixteen hours before the end of the incubation, 1.0 μCi $^3$H-thymidine was added to each well. Plates were harvested with a Tomtec cell harvester and $^3$H-thymidine incorporation determined by using a Perkin Elmer β-scintillation counter.

Each sample was tested for 3 parameters: (1) Treg-cell anergy (Treg cell alone APC); (2) Treg-cell suppression (Treg cell: $CD4^+$T cell with APC); and (3) T-cell proliferation ($CD4^+$ T cell with APC). As expected from our previously published results,[E2,E3] Treg cells alone did not usually proliferate in response to APC stimulus, and we similarly found states of Treg-cell anergy in all 4 groups (SA, FA, SNA, FNA; data not shown). None of the subjects had taken oral steroids within 1 month of blood draw, a treatment that we have found can lead to Treg-cell functional changes if they are taken within 4 days of sampling.[E2,E3] Finally, no subjects with an active infection within 2 weeks before blood collection were included in these assays.

## Chemotaxis assays

Specific migration of purified Treg cells from each subject (FACES, n = 71; and SNA, n = 40) was evaluated with a 5-μm-pore Transwell system (Corning Costar, Lowell, Mass). Flow-sorted, live, purified Treg cells ($2.5 \times 10^5$) were suspended in 100 μL RPMI-1640 medium with 0.5% BSA and added to the top compartment. Primary control HBECs (normal HBECs; Promocell, Heidelberg, Germany) were grown to 80% confluence ($4 \times 10^5$ cells/mL) in the bottom well in 600 μL BEC media. Migration was assayed for 2.5 hours at 37°C; Transwell inserts were removed. Migrated Treg cells were enumerated with an automatic cell counter (Beckman Coulter, Fullerton, Calif), and this approach was also used to quantify the starting cell populations. To assess background migration, we performed negative controls (Treg cells in top, media only in bottom) for each sample, and positive controls (Treg cells placed in bottom in media, no BEC) were run for each sample. We have shown

that minimal migration of Treg cells occurs from top to bottom of the Transwell.[E2,E3] For blocking studies, 20 μL anti-CCR4, anti-CCR8, or anti-CCR7 antibodies (clones 205410, 191704, or 150503, respectively; R&D Systems, Minneapolis, Minn), which had previously been titrated to effect and used as per the manufacturer's instructions, or 20 μL of the isotype control (R&D Systems) or media alone for 30 minutes were placed with the Treg cells in the top Transwell.

## Transcript expression studies

Total RNA was isolated by using RNeasy kits according to the manufacturer's protocol (Qiagen, Valencia, Calif). For cDNA synthesis, 500 ng total RNA was transcribed with cDNA transcription reagents (Applied Biosystems, Foster City, Calif) by using random hexamers, according to the manufacturer's protocol. Real-time PCR was performed for Foxp3, IL-10, TGF-β, CD25, CD45 isoforms, RO and RA, and signal transducer and activator of transcription 5 by using oligonucleotide primers and other reagents purchased from Applied Biosystems. All assays were performed in triplicate.

## Cytometry studies

Flow-cytometric analysis was performed by using an LSRII digital flow cytometer (BD Biosciences) equipped with 4 lasers (405, 488, 535, 633 nm), 2 light scatter detectors (yielding forward and side scatter data), and 18 fluorescent detectors for analysis. A phenotyping method was used with up to 13 colors of fluorescently conjugated antibodies (LSRII; BD Biosciences). The stains included Live/Dead, CD4, CD25, CD127, and Foxp3. The method is demonstrated in Fig E3. Acquisition was controlled using the DiVa software (BD Biosciences). Collected data were exported to FlowJo software for analysis (Treestar, Ashland, Ore). To standardize signal output by the cytometer before each session, we ran a thorough calibration procedure by using a standard set of multicolor fluorescence beads.

## Methylation studies of Foxp3 DNA

Treg cells and conventional $CD4^+$ T cells were purified as described, and genomic DNA was isolated by using a DNA extraction kit (Qiagen). The DNA was denatured, modified with sodium metabisulfite, purified, and desulfonated by using a CpGenome Fast DNA modification kit (Chemicon International, Temecula, Calif). The following disulfite oligonucleotide primers were designed with MethPrimer software and used in this analysis: Foxp3-promoter-CpGs-forward: TATAATTAAGAAAAGGAGAAATAT-AGAGAG, Foxp3-promoter-CpGs-reverse: TCAACCTAACTTATAAAAAACTATCAC, Foxp3-intron-CpGs-forward: TTGGGTTAAGTTTGTTGTAGGATAG, and Foxp3-intron-CpGs-R reverse: ATCTAAACCCTATTATCACAA. DNA was then sequenced (SayoBiotech, Palo Alto, Calif).

### REFERENCES

E1. Margolis HG, Mann JK, Lurmann FW, Mortimer KM, Balmes JR, Hammond SK, et al. Altered pulmonary function in children with asthma associated with highway traffic near residence. Int J Environ Health Res 2009;19: 139-55.

E2. Nguyen KD, Fohner A, Booker JD, Dong C, Krensky AM, Nadeau KC. XCL1 enhances regulatory activities of CD4+ CD25(high) CD127(low/-) T cells in human allergic asthma. J Immunol 2008;181:5386-95.

E3. Nguyen KD, Vanichsarn C, Fohner A, Nadeau KC. Selective deregulation in chemokine signaling pathways of CD4+CD25(hi)CD127(lo)/(-) regulatory T cells in human allergic asthma. J Allergy Clin Immunol 2009;123:933-9, e910.

E4. Nguyen KD, Vanichsarn C, Nadeau KC. Increased cytotoxicity of CD4+ invariant NKT cells against CD4+CD25hiCD127lo/- regulatory T cells in allergic asthma. Eur J Immunol 2008;38:2034-45.

E5. Yu GP, Chiang D, Song SJ, Hoyte EG, Huang J, Vanishsarn C, et al. Regulatory T cell dysfunction in subjects with common variable immunodeficiency complicated by autoimmune disease. Clin Immunol 2009;131:240-53.

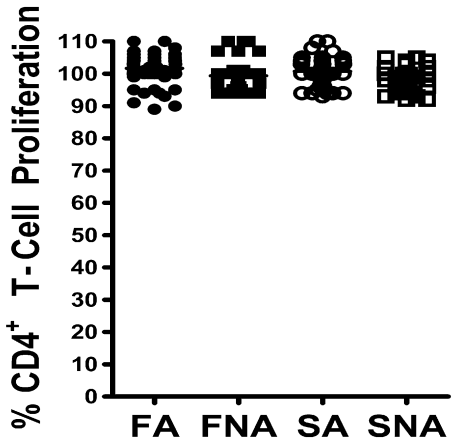


**FIG E1.** *Horizontal bars* are medians; *P* values from Kruskal-Wallis ANOVA and pairwise posttest comparisons via Dunn multiple comparison test. Results are the mean of duplicate experiments for each sample. Conventional $CD4^+$ T-cell proliferation assays without autologous Treg cells added to the culture. The percentage of proliferation represents only responder $CD4^+$ T-cell proliferation among 4 subject groups (FA, n = 71; FNA, n = 30; SA, n = 40; SNA, n = 40).

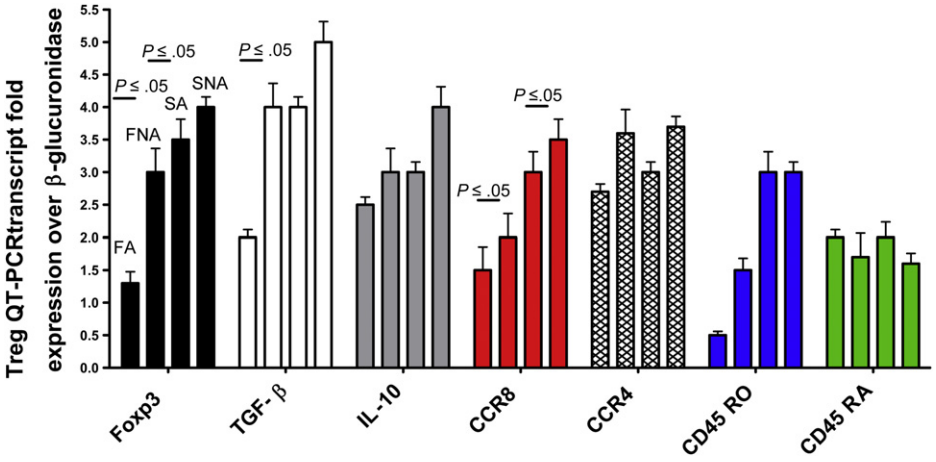


**FIG E2.** Quantitative *(QT)* PCR analysis on purified Treg cells (FA, n = 71; FNA, n = 30; SNA, n = 40; SA, n = 40). Data are presented as a relative fold expression of the candidate gene to the expression of the housekeeping gene β glucuronidase. **P* value ≤.05 based on Kruskal-Wallis 1-way ANOVA using and pairwise posttest comparisons via Dunn multiple comparison test. *Vertical bars* represent SD from the mean.

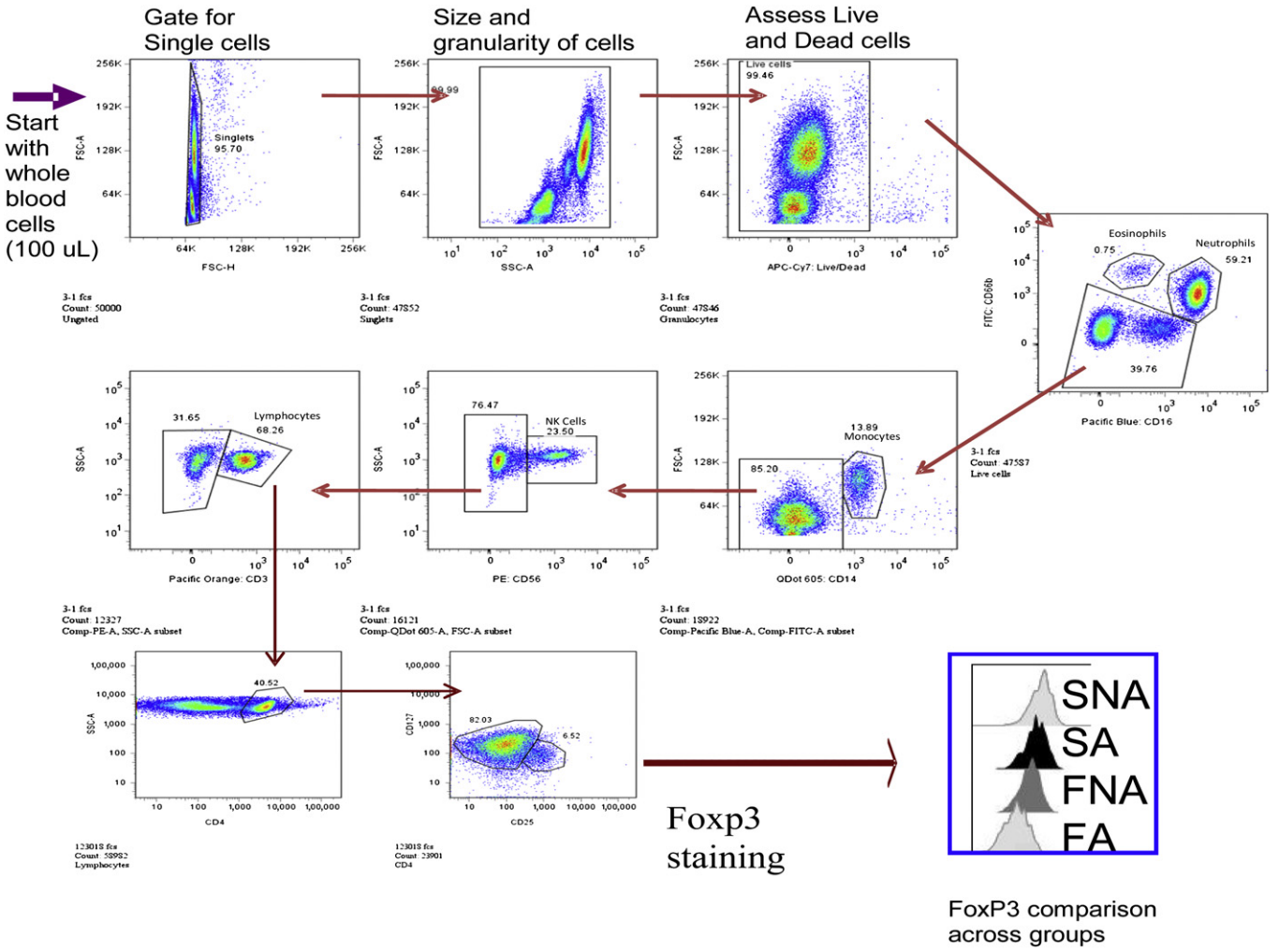


**FIG E3.** Multicolor FACS analysis on gated Treg cells. Whole blood, 100 μL, was directly stained with conjugated fluorescently labeled antibodies for CD4, CD25, CD127 (BD Biosciences), and Foxp3 (BioLegend, clone 259D, Alexa-Fluor 488), of representative subject (SA). A histogram of Foxp3 expression is added for representative SNA, FNA, and FA. *FITC,* Fluorescein isothiocyanate.

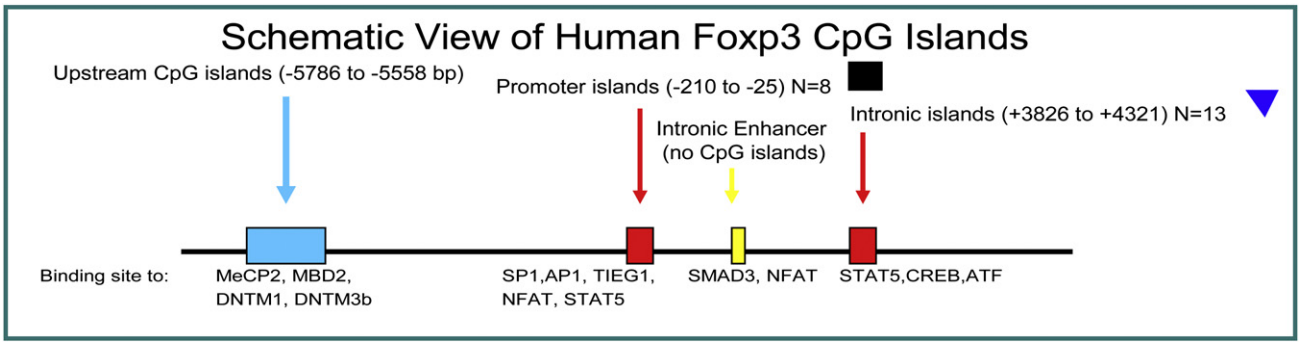


**FIG E4.** Schematic view of human Foxp3 CpG islands. *CpG*, cytosine phosphate guanine; *MeCP2*, methyl CpG binding protein 2; *MBD2*, (methyl-CpG binding domain protein 2)-interacting zinc finger; *DNTM1*, DNA methyl transferase protein 1; *DNTM3b*, DNA methyl transferase protein 3b; *SP1*, stimulator protein 1; *AP1*, activator protein 1; *TIEG1*, TGF-beta-inducible early response gene-1; *NFAT*, nuclear factor and activated T cells; *STAT*, signal transduction and activator of transcription; *SMAD*, small phenotype and mothers against dodecahedron 3; *CREB*, cycle amp response element binding protein; *ATF*, activating transcription factor.

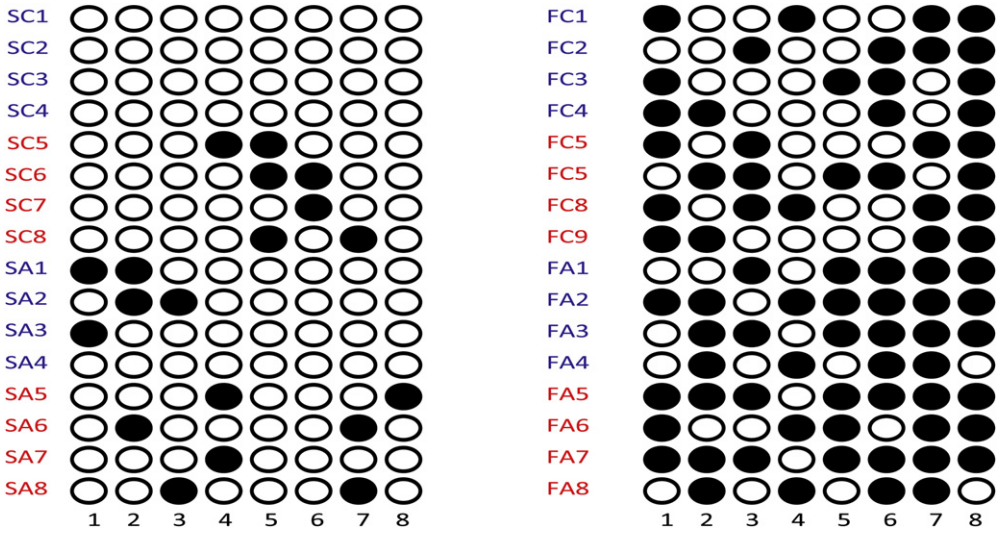


**FIG E5.** Methylation status of CpG islands in Foxp3 promoter region by bisulfite sequencing analysis. Each *line* (differentiate row vs column) represents 1 DNA strand; *open circles,* unmethylated CpG; *filled circles,* methylated CpG. *Circles 1 through 8* represent the 8 CpG islands in the Foxp3 promoter region. Representative subjects determined randomly are shown (*red,* female; *blue,* male). Subjects from Fresno (FA, n = 8; FC [fresno controls], n = 8) were highly methylated compared with those from Stanford (SA, n = 8; SC [stanford controls], n = 8).

**TABLE E1.** Demographics of Fresno asthmatics

| Subject | 2nd hand smoke exp? | Age | Sex | Birth weight (kg) | Age at 1st asthma dxs (a) | Global Initiative for Asthma at t | Skin test | Maternal history of asthma? | History of hospitalization for asthma? | History of emergency room/department visit for asthma? | Percent predicted $FEV_1$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FA1 | N | 17 | M | 3.3 | 3 | 3 | Y | N | Y | Y | 85 |
| FA2 | N | 16 | M | 3.4 | 5 | 2 | Y | N | N | N | 77 |
| FA3 | Y | 15 | F | 3.5 | 5 | 2 | Y | Y | N | N | 91 |
| FA4 | N | 18 | M | 3.5 | 6 | 2 | Y | N | N | N | 75 |
| FA5 | Y | 17 | F | 3.8 | 3 | 1 | Y | Y | N | N | 88 |
| FA6 | N | 16 | F | 2.9 | 0 | 2 | N | Y | N | N | 99 |
| FA7 | N | 16 | F | 2.2 | 5 | 1 | N | N | N | N | 117 |
| FA8 | N | 18 | F | 3.7 | 6 | 2 | Y | N | Y | Y | 112 |
| FA9 | N | 15 | M | 3.4 | 4 | 2 | Y | N | N | N | 98 |
| FA10 | N | 14 | M | 3.3 | 1 | 1 | Y | N | Y | NA | 106 |
| FA11 | N | 15 | M | 3.2 | 3 | 1 | Y | N | N | N | 100 |
| FA12 | N | 16 | M | 2.4 | 8 | 3 | Y | Y | N | N | 95 |
| FA13 | Y | 16 | M | 3.7 | 6 | 2 | Y | Y | N | N | 110 |
| FA14 | N | 14 | M | 3.4 | 0 | 4 | Y | N | N | N | 87 |
| FA15 | N | 17 | F | 3.7 | 7 | 3 | N | Y | N | N | 83 |
| FA16 | N | 13 | M | 3.2 | 2 | 2 | Y | Y | N | N | 98 |
| FA17 | N | 17 | M | NA | 0 | 2 | Y | Y | Y | NA | 100 |
| FA18 | N | 13 | M | 3.1 | 3 | 2 | Y | Y | N | N | 87 |
| FA19 | N | 13 | F | 3.2 | 0 | 2 | N | Y | N | N | 83 |
| FA20 | N | 15 | M | NA | 6 | 2 | Y | N | Y | NA | 91 |
| FA21 | N | 15 | M | 3.2 | 2 | 1 | Y | N | N | N | 107 |
| FA22 | N | 16 | F | 3.5 | 4 | 2 | Y | N | N | N | 78 |
| FA23 | N | 12 | F | 3.8 | 3 | 3 | Y | Y | Y | NA | 99 |
| FA24 | N | 16 | F | 2.7 | 5 | 2 | NA | N | N | N | 66 |
| FA25 | Y | 14 | F | 3.9 | 7 | 1 | N | N | N | N | 100 |
| FA26 | N | 12 | M | 4.2 | 6 | 2 | Y | N | N | N | 68 |
| FA27 | N | 15 | M | NA | 6 | 2 | Y | N | N | N | 105 |
| FA28 | N | 15 | M | 3.2 | 5 | 1 | N | N | N | N | 73 |
| FA29 | N | 14 | M | 3.7 | 6 | 1 | N | N | N | N | 92 |
| FA30 | N | 16 | F | 3.7 | 0 | 2 | Y | N | Y | NA | 96 |
| FA31 | N | 15 | M | 3.5 | 3 | 2 | Y | N | Y | NA | 103 |
| FA32 | N | 16 | M | 4.4 | 9 | 2 | Y | N | N | N | 114 |
| FA33 | N | 17 | M | 4.5 | 5 | 1 | Y | Y | N | N | 102 |
| FA34 | N | 15 | M | 4 | 1 | 1 | N | N | Y | NA | 104 |
| FA35 | N | 15 | F | 3 | 3 | 2 | N | NA | N | N | 99 |
| FA36 | N | 11 | F | NA | 5 | 2 | Y | N | N | N | 85 |
| FA37 | N | 16 | F | 2.8 | 10 | 1 | Y | Y | N | N | 100 |
| FA38 | N | 11 | M | 2.8 | 5 | 2 | Y | Y | N | N | 93 |
| FA39 | N | 14 | F | 3.5 | 3 | 2 | N | N | N | N | 104 |
| FA40 | N | 14 | F | 3 | 7 | 2 | Y | Y | N | N | 108 |
| FA41 | N | 13 | M | 3.2 | 7 | 2 | NA | N | N | N | 74 |
| FA42 | N | 16 | F | NA | NA | 3 | N | N | N | N | 85 |
| FA43 | N | 13 | M | NA | 7 | 1 | N | N | N | N | 103 |
| FA44 | N | 11 | M | 4.2 | 2 | 3 | Y | N | N | N | 100 |
| FA45 | N | 15 | M | 3.3 | 9 | 2 | Y | Y | N | N | 101 |
| FA46 | N | 13 | M | 2.8 | 8 | 2 | N | N | N | N | 76 |
| FA47 | Y | 11 | F | 3.9 | 6 | 3 | NA | Y | N | N | 119 |
| FA48 | N | 11 | F | 2.9 | 2 | 2 | N | N | N | N | 79 |
| FA49 | N | 14 | M | 3.1 | 4 | 2 | N | Y | N | N | 110 |
| FA50 | N | 10 | F | 3.4 | 6 | 2 | NA | N | N | N | 87 |
| FA51 | Y | 13 | M | NA | 3 | 2 | Y | N | Y | NA | 72 |
| FA52 | N | 13 | M | 3.3 | 3 | 1 | Y | Y | N | N | 90 |
| FA53 | N | 11 | M | 3 | 5 | 3 | N | N | N | N | 76 |
| FA54 | N | 13 | M | 3.7 | 8 | 1 | Y | N | N | N | 90 |
| FA55 | N | 14 | F | NA | 10 | 2 | N | N | N | N | 72 |
| FA56 | N | 9 | F | NA | 0 | 1 | Y | NA | N | N | 89 |
| FA57 | N | 10 | F | 3.1 | 3 | 1 | N | N | N | N | 96 |
| FA58 | N | 12 | M | 3.3 | 1 | 2 | Y | N | N | N | 81 |
| FA59 | Y | 13 | F | 3.5 | 6 | 1 | Y | Y | N | N | 128 |
| FA60 | N | 16 | F | NA | 11 | 2 | N | Y | N | N | 83 |

*(Continued)*

**TABLE E1**. *(Continued)*

| Subject | 2nd hand smoke exp? | Age | Sex | Birth weight (kg) | Age at 1st asthma dxs (a) | Global Initiative for Asthma at t | Skin test | Maternal history of asthma? | History of hospitalization for asthma? | History of emergency room/department visit for asthma? | Percent predicted $FEV_1$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FA61 | N | 14 | M | 3.4 | 2 | 3 | Y | N | N | N | 81 |
| FA62 | N | 11 | M | 3.2 | 5 | 1 | NA | N | N | N | 98 |
| FA63 | N | 12 | M | NA | 2 | 1 | Y | Y | N | N | 74 |
| FA64 | N | 11 | M | 3.3 | 5 | 1 | N | NA | N | N | 99 |
| FA65 | N | 11 | M | 2.4 | 5 | 2 | N | Y | N | N | 88 |
| FA66 | N | 12 | F | 3.5 | 5 | 1 | N | N | N | N | 92 |
| FA67 | N | 14 | M | NA | 3 | 4 | N | N | N | N | 88 |
| FA68 | N | 9 | F | 3.9 | 2 | 1 | Y | N | N | N | 104 |
| FA69 | N | 12 | F | 3.3 | 4 | 2 | Y | Y | N | N | 97 |
| FA70 | N | 9 | F | 3.4 | 1 | 1 | Y | Y | N | N | 90 |
| FA71 | N | 9 | M | NA | 6 | 4 | NA | Y | N | M | 80 |

*F,* Female; *M,* male; *N,* no; *NA,* not applicable; *t*, time of spirometry; *Y,* yes.

**TABLE E2.** Demographics of Stanford asthmatics

| Subject | 2nd hand smoke exp? | Age | Sex | BW (kg) | Age at 1st asthma dxs (a) | Gina at t | Skin test | Mat hx of asthma? | Hx of hosp for asthma? | Hx of ER visit for asthma? | pp $FEV_1$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SA1 | N | 11 | M | 3.1 | 8 | 3 | Y | Y | Y | Y | 75 |
| SA2 | N | 12 | M | 3.4 | 8 | 3 | Y | Y | N | Y | 76 |
| SA3 | N | 16 | F | 4 | 11 | 2 | Y | Y | Y | N | 80 |
| SA4 | N | 17 | F | 3.6 | 7 | 2 | N | N | N | Y | 83 |
| SA5 | N | 9 | F | 2.8 | 6 | 1 | Y | Y | Y | N | 93 |
| SA6 | N | 7 | M | 2.7 | 6 | 1 | N | Y | N | Y | 97 |
| SA7 | Y | 18 | M | 3 | 7 | 1 | N | N | N | N | 99 |
| SA8 | N | 9 | F | 3.5 | 7 | 1 | Y | N | Y | N | 100 |
| SA9 | N | 10 | M | 2.6 | 9 | 1 | Y | N | N | N | 104 |
| SA10 | N | 13 | F | NA | 11 | 1 | N | N | N | N | 105 |
| SA11 | N | 15 | M | 4.1 | 7 | 1 | N | N | N | N | 110 |
| SA12 | N | 15 | M | 3.7 | 12 | 1 | Y | Y | N | N | 94 |
| SA13 | Y | 9 | F | 3.2 | 7 | 1 | Y | Y | Y | N | 96 |
| SA14 | N | 10 | F | 3.3 | 9 | 1 | N | Y | N | N | 88 |
| SA15 | N | 14 | F | NA | 7 | 1 | N | N | N | N | 93 |
| SA16 | N | 12 | M | 2.9 | 7 | 1 | N | Y | N | Y | 91 |
| SA17 | N | 18 | F | 3.4 | 8 | 1 | Y | N | N | N | 94 |
| SA18 | N | 11 | F | 3.8 | 8 | 1 | Y | Y | Y | N | 85 |
| SA19 | N | 9 | M | 2.8 | 8 | 1 | N | N | N | N | 88 |
| SA20 | N | 8 | F | 2.7 | 12 | 1 | Y | N | N | Y | 90 |
| SA21 | N | 8 | M | 3 | 6 | 1 | Y | N | Y | Y | 120 |
| SA22 | N | 9 | M | 3.2 | 6 | 3 | Y | Y | N | N | 79 |
| SA23 | N | 11 | M | 3.6 | 7 | 2 | Y | Y | Y | Y | 82 |
| SA24 | Y | 18 | F | 3.7 | 6 | 1 | Y | N | Y | Y | 90 |
| SA25 | N | 17 | F | 3 | 8 | 1 | Y | N | Y | Y | 92 |
| SA26 | N | 10 | F | 2.6 | 6 | 1 | Y | N | Y | Y | 99 |
| SA27 | N | 9 | M | 3.1 | 9 | 1 | Y | Y | N | Y | 100 |
| SA28 | N | 12 | M | 2.8 | 11 | 1 | Y | Y | Y | Y | 104 |
| SA29 | N | 14 | F | 3.5 | 13 | 1 | Y | N | N | N | 106 |
| SA30 | N | 11 | F | 4 | 8 | 1 | Y | Y | N | N | 108 |
| SA31 | N | 15 | F | 3.5 | 7 | 1 | Y | N | N | N | 110 |
| SA32 | N | 16 | M | 2.7 | 11 | 1 | Y | N | N | N | 110 |
| SA33 | Y | 18 | M | 3 | 6 | 1 | Y | N | Y | Y | 106 |
| SA34 | N | 9 | M | 4.1 | 7 | 1 | N | Y | Y | Y | 94 |
| SA35 | N | 12 | F | 4.2 | 8 | 1 | Y | Y | N | N | 90 |
| SA36 | N | 10 | F | NA | 9 | 2 | Y | Y | N | N | 86 |
| SA37 | N | 8 | M | NA | 6 | 1 | Y | N | Y | N | 88 |
| SA38 | N | 17 | F | 2.9 | 12 | 3 | Y | Y | Y | Y | 77 |
| SA39 | N | 16 | F | 3 | 13 | 2 | Y | Y | N | N | 80 |
| SA40 | N | 16 | M | 3.7 | 8 | 1 | Y | N | Y | Y | 85 |

*F,* Female; *M,* male; *N,* no; *NA,* not applicable; *t,* time of spirometry; *Y,* yes.