

# Natural regulatory T cells in isolated early responders compared with dual responders with allergic asthma

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**Background:** Natural regulatory T (Treg) cells are implicated in the regulation of the inflammatory response in patients with allergic asthma.

**Objectives:** We sought to determine changes in Treg cell numbers in the airways and peripheral blood of isolated early responder (IER) versus dual responder (DR) subjects with mild allergic asthma before and after allergen challenge.

**Methods:** Induced sputum was collected from 22 subjects with allergic asthma (10 IERs and 12 DRs) and peripheral blood collected from 8 DRs with allergic asthma at 0, 7, and 24 hours after allergen challenge. Treg cells were identified by using fluorescently labeled antibodies to CD4 and forkhead box protein 3 and enumerated by using flow cytometry.

**Results:** There was a significant increase in the percentage of sputum CD4<sup>+</sup> cells 24 hours after allergen challenge in both IERs and DRs. The percentage of sputum Treg cells significantly decreased 24 hours after challenge in DRs but not IERs. This change was significantly correlated with the magnitude of the late asthmatic response. There was also a significant increase in the absolute number of sputum CD4<sup>+</sup> cells and Treg cells at 24 hours in DRs only. The ratio of the number of Treg cells to CD4<sup>+</sup> cells at 24 hours was significantly smaller in DRs compared with that in IERs. None of the above changes were observed in peripheral blood.

**Conclusions:** DRs exhibit a diminished percentage of airway Treg cells after allergen challenge that is not observed in IERs and a significantly lower ratio of Treg cells to CD4<sup>+</sup> cells, which might contribute to the development of the late asthmatic response. (*J Allergy Clin Immunol* 2013;■■■■:■■■-■■■.)

**Key words:** Allergic asthma, induced sputum, CD4<sup>+</sup> T cells, late asthmatic responses, regulatory T cells

Allergic asthma occurs as a result of an inappropriate immune response to innocuous inhaled allergens. The immune and inflammatory processes of this response are complex and include evidence for impaired regulation by regulatory T (Treg) cells. Treg cells, formerly known as suppressor T cells, are immunosuppressive and immunomodulatory T cells that play a role in the maintenance of self-tolerance and immune homeostasis and play a crucial regulatory role in human diseases, such as allergy, cancer, and autoimmune disease.<sup>1-3</sup> They can be classified as natural Treg cells, which develop in the thymus, or induced Treg cells, which develop in the periphery on antigen encounter.<sup>4</sup> Recently, lung tissue macrophages have been shown to have the ability to generate induced Treg cells.<sup>5</sup> Human Treg cells were initially defined as the CD4<sup>+</sup>CD25<sup>+</sup> lymphocyte population.<sup>6,7</sup> Subsequently, forkhead box protein 3 (Foxp3), a forkhead family transcription factor, was identified as a master control gene for Treg cell development, function, and homeostasis.<sup>8,9</sup> Thus natural Treg cells are typically defined as T helper cells that express CD25 and Foxp3 (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells) and induced Treg cells as T<sub>H</sub>1 helper cells that express IL-10 (CD4<sup>+</sup>IL-10<sup>+</sup> cells) or T<sub>H</sub>3 helper cells that express TGF- $\beta$  (CD4<sup>+</sup>TGF- $\beta$ <sup>+</sup> cells). Treg cells exert their suppressive/inhibitory effects through a variety of mechanisms, including cell-cell contact, competition for growth factors, cytotoxicity, and secretion of inhibitory cytokines.

Evidence exists for an immunoregulatory role for Treg cells in animal models of allergic airway responses,<sup>10,11</sup> and this is increasingly supported by clinical evidence. Treg cells are deficient numerically and functionally in asthmatic compared with healthy subjects,<sup>12</sup> and the failure to properly regulate immune responses in asthmatic subjects might be attributable to an insufficiency in tolerance mechanisms. This has led to the hypothesis that in patients with allergic asthma, the Treg cell response is insufficient to control the T-cell effector (T<sub>H</sub>2-induced) inflammatory response. To examine this hypothesis, we have evaluated the absolute numbers and percentages of Treg cells as a proportion of effector T cells in the airways and peripheral blood before and after inhaled allergen challenge in atopic asthmatic subjects. We reasoned that atopic asthmatic subjects, who have a late-phase asthmatic response and greater allergen-induced airway inflammation,<sup>13</sup> would have fewer airway Treg cells after allergen inhalation challenge.

## METHODS

### Subjects

Thirty subjects with very mild allergic asthma (10 isolated early responders [IERs] and 20 dual responders [DRs]) between the ages of 18 and 60 years

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**Abbreviations used**

DR: Dual responder  
 EAR: Early asthmatic response  
 FACS: Fluorescence-activated cell sorting  
 Foxp3: Forkhead box protein 3  
 IER: Isolated early responder  
 LAR: Late asthmatic response  
 Treg: Regulatory T

were enrolled (Table 1). All were atopic on the basis of 1 or more skin wheal-and-flare responses to common aeroallergens and had FEV<sub>1</sub> of 70% or greater of predicted value and baseline methacholine PC<sub>20</sub> (the provocative concentration of methacholine causing a 20% decrease in FEV<sub>1</sub>) of 32 mg/mL or less. All subjects had no other lung disease or chronic illness and no lower respiratory tract infection or worsening of asthma for 6 weeks before screening. Subjects had not been using inhaled corticosteroids for at least 1 year, had infrequent use of  $\beta_2$ -agonists for treatment of asthma (less than daily), and refrained from  $\beta_2$ -agonists and caffeinated beverages at least 8 hours before laboratory visits. Subjects were excluded if they were pregnant, current smokers, or exsmokers with a more than 10-pack-year history. IERs were defined as those having only an early asthmatic response (EAR; ie, acute decrease in FEV<sub>1</sub>  $\geq 20\%$  within 2 hours after allergen challenge). DRs were defined as those subjects having both an EAR and a late asthmatic response (LAR; ie, decrease in FEV<sub>1</sub>  $\geq 15\%$  between 3 and 7 hours after allergen challenge). Eight subjects (all DRs) provided peripheral blood for Treg cell analysis. The study was approved by the hospital's research ethics review board, and all subjects provided written informed consent.

**Study design**

Subjects attended the study period on 3 consecutive days. On day 1, a medical history and physical examination were performed, and subjects underwent a skin prick test, spirometry, methacholine inhalation challenge, blood collection, and sputum induction. On day 2, subjects underwent an inhaled allergen challenge, and spirometry was measured up to 7 hours after challenge. Sputum was induced and blood was collected at 7 hours after challenge. On day 3, 24 hours after allergen inhalation challenge, subjects performed spirometry, methacholine inhalation challenge, blood collection, and sputum induction.

**Methacholine inhalation challenge**

Methacholine inhalation challenge was performed, as previously described by Cockcroft et al,<sup>14</sup> by using tidal breathing from a Wright nebulizer. The test was terminated when a decrease in FEV<sub>1</sub> of at least 20% of the baseline value occurred.

**Allergen inhalation challenge**

Allergen inhalation was performed, as described by O'Byrne et al.<sup>15</sup> The concentration of allergen extract used for inhalation was determined from a formula described by Cockcroft et al.<sup>16</sup> After allergen administration, FEV<sub>1</sub> was then measured at regular intervals until 7 hours after allergen inhalation. The maximum percentage decrease in FEV<sub>1</sub> was recorded for the EAR and LAR. The EAR was identified by a decrease in FEV<sub>1</sub> of 20% or greater from baseline within 2 hours after allergen, and an LAR was identified by a decrease in FEV<sub>1</sub> of 15% or greater from baseline between 3 and 8 hours after allergen.

**Sputum induction and processing**

Sputum was induced and processed according to the method described by Pizzichini et al.<sup>17</sup> The total cell count was determined with a Neubauer hemocytometer chamber (Hausser Scientific, Blue Bell, Pa) and expressed

as the number of cells per gram of sputum. Cells were prepared on 2 glass slides and subsequently stained with Diff-Quik (American Scientific Products, McGaw Park, Ill). Duplicate differential counts (400 cells per slide) were averaged. The remaining sputum cells were used for measurement of Treg cell numbers.

**Blood collection and processing**

Venous blood was withdrawn into vacuum blood collection tubes containing lithium heparin. Blood was diluted 1:1 with McCoy 5A (Gibco, Carlsbad, Calif) and layered over Accu-Prep density gradient medium (Accurate Chemical and Scientific Corp, New York, NY). Layered blood was subjected to density gradient separation (centrifugation at 2000 rpm for 20 minutes at room temperature). Mononuclear cells were removed and washed with McCoy 5A (centrifugation at 1500 rpm for 10 minutes at 4°C). Cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 0.1% sodium azide and 0.5% BSA) and subjected to a Treg cell staining protocol.

**Staining**

Freshly processed sputum cells were divided into 0.6 to 1  $\times 10^6$  cells per tube. Cells were washed with FACS buffer (centrifugation at 1500 rpm for 10 minutes at 4°C), resuspended in 80  $\mu$ L of 5% normal mouse block, and incubated on ice in the dark for 10 minutes. This was followed by surface staining with the following fluorescent antibodies: Pacific Blue-CD3 (eBioscience, San Diego, Calif), PE-Cy7-CD4 (Beckman Coulter, Fullerton, Calif), and relevant isotype controls (BD PharMingen, San Jose, Calif). Cells and antibodies were incubated for 30 minutes on ice in the dark. Cells were then washed with FACS buffer and resuspended in 1 mL of Fix/Perm solution (eBioscience) for 30 minutes at 4°C in the dark. Cells were washed with Perm buffer (eBioscience), resuspended in 100  $\mu$ L of 5% normal rat block, and incubated for 15 minutes at 4°C in the dark. This was followed by intracellular staining with fluorescein isothiocyanate-Foxp3 (eBioscience) and relevant isotype control and incubated for 30 minutes at 4°C in the dark. Cells were then washed twice with Perm buffer, resuspended in 500  $\mu$ L of Cytofix (1% paraformaldehyde, BD Biosciences), and kept at 4°C in the dark until flow cytometric acquisition.

Staining of peripheral blood cells was performed as per the sputum protocol by using the following antibodies: surface antibodies, CD3-AmCyan, CD4-Pacific Blue, CD127-fluorescein isothiocyanate, and relevant isotype controls; intracellular antibody, FoxP3-allophycocyanin and relevant isotype control (all from eBioscience).

**Flow cytometry**

Data were acquired by using a 15-color LSRII flow cytometer equipped with 3 lasers (Becton Dickinson Instrument Systems) with FACSDiva acquisition software (Becton Dickinson BioSciences, Mississauga, Ontario, Canada). For sputum, as many CD4<sup>+</sup> events were collected as possible. For peripheral blood, at least 20,000 CD4<sup>+</sup> events were collected. Collected data were analyzed by using FlowJo flow cytometric analysis software (Treestar, Ashland, Ore). Our Treg cell gating strategy is outlined in Fig 1. Briefly, a gate was placed on the CD3<sup>+</sup> population. A CD3 versus CD4 dot plot was generated by using only cells from the CD3<sup>+</sup> gate. Double-positive CD3<sup>+</sup>/CD4<sup>+</sup> cells were analyzed on a dot plot of CD4 versus isotype control for Foxp3. A threshold was set at 2%, and this was then applied to a dot plot of CD4 versus Foxp3. Cells to the right of the 2% threshold were deemed positive for Foxp3.

**Determination of absolute number**

It is difficult to obtain the absolute number of Treg cells in induced sputum because there are so few T cells to enumerate on sputum slides. Thus the number of Treg cells was divided by the total number of cells in the total cell gate on the CD3 versus side scatter plot to quantify absolute numbers in sputum (Fig 2). This percentage was then multiplied by the absolute cell

TABLE I. Subjects' characteristics

| Sex                 | Age (y)     | FEV <sub>1</sub><br>(% predicted) | Mch PC <sub>20</sub><br>(mg/mL) | Antigen inhaled           | EAR<br>(% decrease in FEV <sub>1</sub> ) | LAR<br>(% decrease in FEV <sub>1</sub> ) |
|---------------------|-------------|-----------------------------------|---------------------------------|---------------------------|------------------------------------------|------------------------------------------|
| Sputum IER          |             |                                   |                                 |                           |                                          |                                          |
| F                   | 22          | 82                                | 1.87                            | Cat                       | 41.5                                     | 9.4                                      |
| F                   | 19          | 77                                | 0.52                            | Cat                       | 40.5                                     | 4.7                                      |
| M                   | 50          | 106                               | 10.08                           | HDM DP                    | 27.5                                     | 10.7                                     |
| M                   | 19          | 86                                | 21.6                            | HDM DP                    | 24.5                                     | 9.9                                      |
| F                   | 22          | 91                                | 4                               | Ragweed                   | 38.3                                     | 3.3                                      |
| M                   | 60          | 103.6                             | 7.78                            | Cat                       | 24.0                                     | 13.7                                     |
| F                   | 52          | 86                                | 10.56                           | HDM DP                    | 35.1                                     | 5.4                                      |
| M                   | 60          | 75                                | 15.45                           | HDM DP                    | 36.0                                     | 7.0                                      |
| F                   | 32          | 91                                | 2.07                            | <i>Alternaria</i> species | 46.0                                     | 3.0                                      |
| F                   | 27          | 78                                | 32                              | Ragweed                   | 27.0                                     | 13.0                                     |
| Mean ± SEM          | 36.3 ± 5.44 | 87.5 ± 3.36                       |                                 |                           | 34.0 ± 2.46                              | 8.01 ± 1.23                              |
| Sputum DR           |             |                                   |                                 |                           |                                          |                                          |
| M                   | 26          | 102                               | 3.32                            | Ragweed                   | 46.0                                     | 19.0                                     |
| F                   | 18          | 98                                | 19.6                            | Ragweed                   | 31.0                                     | 28.0                                     |
| M                   | 21          | 101                               | 28.8                            | Grass                     | 36.8                                     | 28.7                                     |
| F                   | 21          | 100                               | 10.29                           | Cat                       | 37.5                                     | 15.3                                     |
| M                   | 50          | 100                               | 5.62                            | HDM DP                    | 39.5                                     | 20.5                                     |
| F                   | 25          | 93                                | 12.44                           | HDM DP                    | 53.6                                     | 15.2                                     |
| M                   | 19          | 103                               | 0.97                            | HDM DP                    | 41.9                                     | 16.3                                     |
| M                   | 18          | 77                                | 0.8                             | Cat                       | 27.9                                     | 45.9                                     |
| M                   | 21          | 110                               | 14.55                           | HDM DP                    | 38.1                                     | 17.9                                     |
| M                   | 22          | 75                                | 0.5                             | HDM DP                    | 44.1                                     | 32.4                                     |
| F                   | 44          | 99                                | 0.74                            | Cat                       | 26.7                                     | 21.7                                     |
| M                   | 61          | 88                                | 8                               | Cat                       | 33.0                                     | 27.0                                     |
| Mean ± SEM          | 28.8 ± 4.17 | 95.5 ± 3.04                       |                                 |                           | 38.0 ± 2.29                              | 23.9 ± 2.59                              |
| Peripheral blood DR |             |                                   |                                 |                           |                                          |                                          |
| M                   | 20          | 94                                | 32                              | Grass                     | 44.0                                     | 22.0                                     |
| M                   | 19          | 70                                | 0.11                            | Cat                       | 28.0                                     | 27.0                                     |
| F                   | 20          | 107                               | 3.6                             | Cat                       | 35.0                                     | 29.0                                     |
| F                   | 22          | 93                                | 0.56                            | Cat                       | 40.0                                     | 17.0                                     |
| F                   | 24          | 89                                | 11.62                           | HDM DP                    | 54.0                                     | 25.0                                     |
| M                   | 41          | 79                                | 1.18                            | Grass                     | 40.0                                     | 23.0                                     |
| F                   | 46          | 89                                | 0.55                            | Cat                       | 45.0                                     | 17.0                                     |
| F                   | 20          | 93                                | 0.14                            | Cat                       | 40.0                                     | 18.0                                     |
| Mean ± SEM          | 26.5 ± 3.78 | 89.2 ± 3.88                       |                                 |                           | 40.8 ± 2.68                              | 22.2 ± 1.63                              |

F, Female; HDM DP, House dust mite (*Dermatophagoides pteronyssinus*); M, male; Mch, methacholine.

number, as determined based on hemacytometer count after sputum processing. In peripheral blood Treg cell numbers were divided by the number of lymphocytes in the lymphocyte gate generated on the forward scatter versus side scatter plot. This percentage was then multiplied by the number of lymphocytes recorded on complete blood count, with the absolute number of cells expressed as cells per milliliter of blood.

## Statistics

Statistical analysis was done with GraphPad Prism5 software (GraphPad Software, La Jolla, Calif). Data with normal distribution were expressed as means ± SEMs. Preallergen challenge versus postallergen challenge comparisons at each time point were performed with unpaired *t* tests. Repeated-measures ANOVA was used for comparisons of baseline, 7-hour, and 24-hour postallergen challenge time points. If the results of ANOVA were significant, then subsequent 2-way comparisons were performed by using Tukey multiple comparison tests. A *P* value of less than .05 was considered significant for all analyses.

## RESULTS

The mean maximal percentage decrease in FEV<sub>1</sub> during the EAR was 34.04% ± 2.46% in IERs and 38.01% ± 2.24% in DRs, whereas the mean maximal percentage decrease in

FEV<sub>1</sub> during the LAR was 8.01% ± 1.23% in IERs and 23.99% ± 2.59% in DRs (*P* < .01, Fig 2 and Table I). Baseline FEV<sub>1</sub> values were not different between the 2 groups.

There were no differences in sputum total cell counts between IERs and DRs at baseline or at 7 and 24 hours after allergen inhalation (Table II); however, the absolute number of CD4<sup>+</sup> cells in sputum significantly increased in DRs, from 80.5 ± 27.89 × 10<sup>3</sup> cells to 372.3 ± 115.2 × 10<sup>3</sup> cells 24 hours after allergen (*P* < .05), but not in IERs (Fig 3, A). Also, the percentage of CD4<sup>+</sup> cells of CD3<sup>+</sup> cells significantly increased from 46.7% ± 3.88% at baseline to 67.0% ± 5.90% in DRs (*P* < .05) and from 43.7% ± 3.94% at baseline to 58.2% ± 6.29% in IERs (*P* < .05) 24 hours after allergen (Fig 3, B).

There were no significant differences in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in sputum between IERs and DRs at baseline; however, the absolute number of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells significantly increased 24 hours after allergen compared with baseline values in induced sputum, but not peripheral blood, in DRs only (*P* < .05; Fig 4, A, and Table III). When expressed as a percentage of total CD4<sup>+</sup> cells, CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell values were significantly lower at 24 hours after allergen in DRs, being 19.88% ± 3.56% at baseline and 8.10% ± 1.27% after allergen

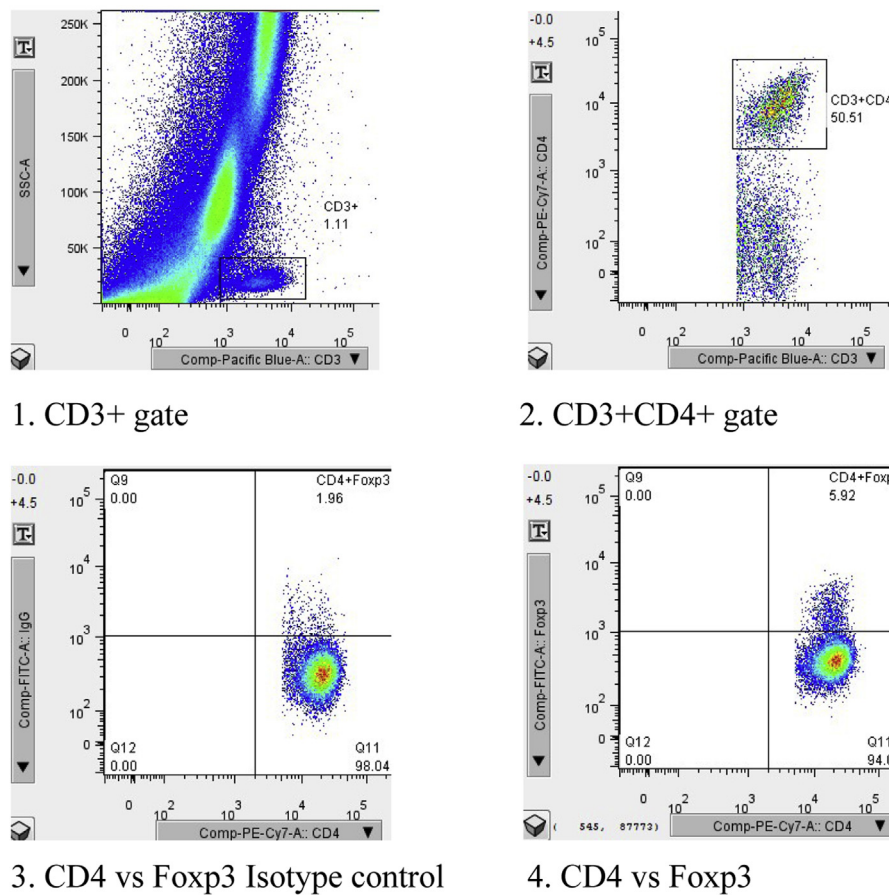
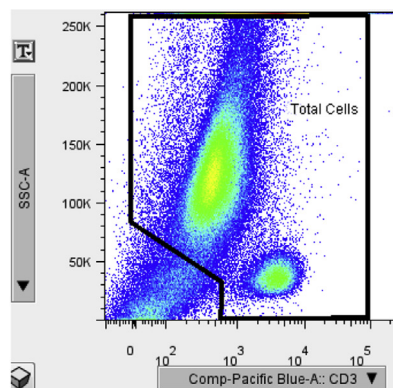
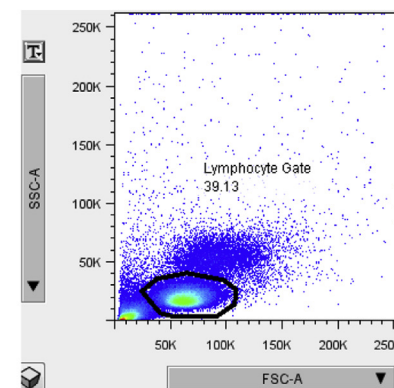
**A** CD4<sup>+</sup>Foxp3<sup>+</sup> Treg gating strategy (representative sputum sample)**B** Flow cytometric gating for the determination of absolute number**i) Sputum****ii) Peripheral Blood**

**FIG 1.** Flow cytometric CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell gating strategy and gating strategy for the determination of absolute numbers in sputum and peripheral blood. **A**, CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell gating strategy (representative sputum sample): 1, CD3<sup>+</sup> gate; 2, CD3<sup>+</sup>CD4<sup>+</sup> gate; 3, CD4 versus Foxp3 isotype control; and 4, CD4 versus Foxp3. **B**, Flow cytometric gating for determination of absolute numbers: *i*, sputum; *ii*, peripheral blood. FSC, Forward scatter; SSC, side scatter.

( $P < .05$ ; Fig 4, B). The ratio of Treg cells/CD4<sup>+</sup> cells at 24 hours after allergen was  $0.08 \pm 0.01$  in DRs and  $0.23 \pm 0.04$  in IERs ( $P < .05$ , Fig 5).

Allergen challenge significantly increased sputum eosinophil and macrophage percentages at 7 and 24 hours after allergen challenge in both early responders and DRs (Table II). There was

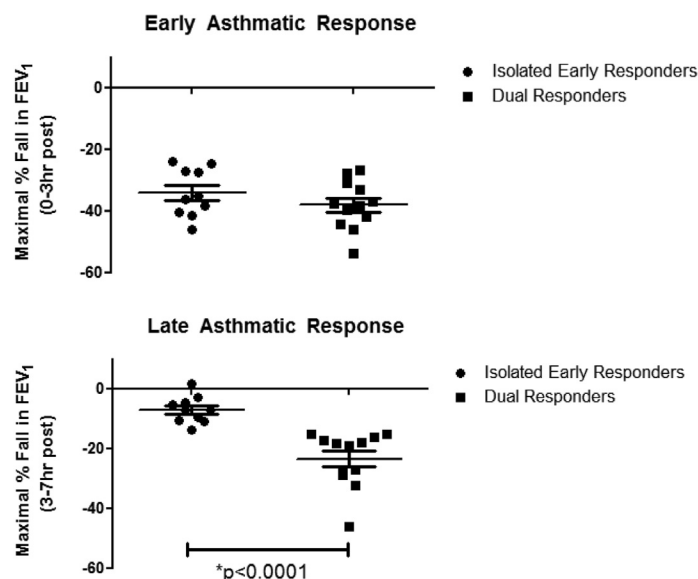


FIG 2. Allergen-induced EARs and LARs.

TABLE II. Allergen-induced changes in sputum inflammatory cells

| Cell type                | Group     | Baseline        | 7 h after allergen      | 24 h after allergen     |
|--------------------------|-----------|-----------------|-------------------------|-------------------------|
| TCC ( $10^6/\text{mL}$ ) | IER       | $4.80 \pm 1.22$ | $6.97 \pm 1.65$         | $7.37 \pm 0.71$         |
|                          | DR        | $4.59 \pm 0.75$ | $9.95 \pm 2.25$         | $10.3 \pm 2.42$         |
|                          | IER vs DR | $P = .88$       | $P = .31$               | $P = .29$               |
| Eosinophils (%)          | IER       | $1.07 \pm 0.31$ | $13.3 \pm 3.34^\dagger$ | $10.3 \pm 1.88^*$       |
|                          | DR        | $1.55 \pm 0.64$ | $16.3 \pm 4.40^*$       | $13.2 \pm 3.85^*$       |
|                          | IER vs DR | $P = .54$       | $P = .59$               | $P = .52$               |
| Neutrophils (%)          | IER       | $49.0 \pm 9.38$ | $64.1 \pm 8.3$          | $67.2 \pm 5.54$         |
|                          | DR        | $36.1 \pm 5.38$ | $64.4 \pm 6.86^\dagger$ | $60.7 \pm 5.81^*$       |
|                          | IER vs DR | $P = .22$       | $P = .98$               | $P = .43$               |
| Macrophages (%)          | IER       | $49.8 \pm 9.35$ | $22.1 \pm 6.55^*$       | $23.6 \pm 4.09^*$       |
|                          | DR        | $62.3 \pm 5.32$ | $19.2 \pm 4.65^\dagger$ | $25.9 \pm 3.96^\dagger$ |
|                          | IER vs DR | $P = .23$       | $P = .71$               | $P = .68$               |

All values are expressed as means  $\pm$  SEMs. Lymphocytes were not detected and therefore not enumerated on the sputum differential slides.

TCC, Total cell count.

\* $P < .05$  versus baseline.

$^\dagger P < .01$  versus baseline.

no significant difference in eosinophil or macrophage percentages between the groups at baseline or 7 and 24 hours after allergen (Table II). Neutrophil values significantly increased in DRs only at 7 and 24 hours after allergen inhalation (Table II). The change in the percentage of  $\text{CD4}^+\text{Foxp3}^+$  Treg cells was significantly correlated with the LAR ( $r = 0.55$ ,  $P = .008$ , Fig 6).

## DISCUSSION

For the first time, this study has demonstrated a decreased percentage of  $\text{CD4}^+\text{Foxp3}^+$  Treg cells in induced sputum 24 hours after allergen inhalation in DRs, but not in IERs, with allergic asthma. In addition, the ratio of  $\text{CD4}^+\text{Foxp3}^+$  Treg cells to total  $\text{CD4}^+$  cells was significantly lower in DRs at 24 hours after allergen when compared with that in IERs, and the change in Treg cell percentages was correlated to the magnitude of the LAR.

Other studies have evaluated Treg cell numbers and function in peripheral blood, bronchoalveolar lavage fluid, or both but not in

sputum. Induced sputum has an advantage in that the kinetics of change in airway cell numbers can be studied more readily than using BALF. Hartl et al<sup>12</sup> reported reduced  $\text{CD4}^+\text{CD25}^{\text{hi}}$  T-cell numbers and function, as well as reduced Foxp3 mRNA, in BALF, but not peripheral blood, from patients with pediatric asthma compared with those seen in children with cough but no asthma. In addition, Thunberg et al<sup>18</sup> described increases in  $\text{CD4}^+\text{Foxp3}^+$  cell numbers in BALF from subjects with mild asthma 24 hours after allergen inhalation compared with that before allergen inhalation but not in peripheral blood; however, this study did not compare subjects with isolated early and dual asthmatic responses after inhaled allergen. Smyth et al<sup>19</sup> reported an increased number of  $\text{CD4}^+\text{Foxp3}^+$  Treg cells and  $\text{CD4}^+$  cytotoxic T lymphocyte-associated antigen 4–positive Treg cells in the BALF of asthmatic subjects, with the highest numbers being observed in those with moderate-to-severe asthma. Also, Sjaheim et al<sup>20</sup> described higher numbers of airway Treg cells, together with effector T cells, activated  $\text{CD8}^+$  cells, and activated  $\text{CD4}^+$  T cells, in patients with occupational asthma. Afshar et al<sup>21</sup>

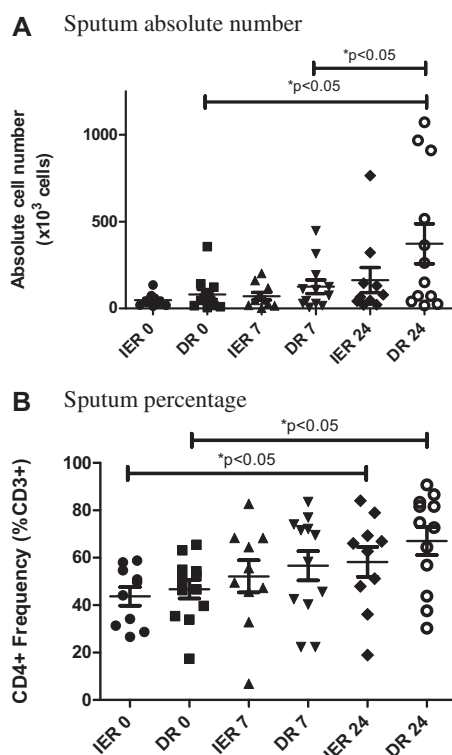


**FIG 3.** Changes in CD4<sup>+</sup> cell absolute numbers and percentages. **A**, Sputum absolute number. **B**, Sputum percentage.

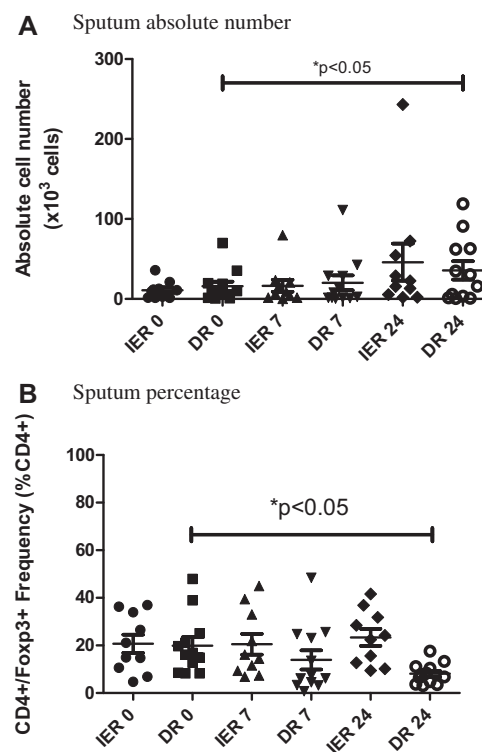


**FIG 4.** Changes in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell absolute numbers and percentages. **A**, Sputum absolute number. **B**, Sputum percentage.

demonstrated that allergic asthmatic subjects had an increase in CCR4-expressing Treg cell numbers in BALF after segmental allergen challenge. Finally, Moniuszko et al<sup>22</sup> compared the number of circulating CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> cells in peripheral blood, but not in the airways, of atopic subjects after allergen inhalation and compared IERs, DRs, and nonresponders. The investigators demonstrated a significant decrease in CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> cell numbers at 6 and 24 hours after allergen, but this was only consistently seen in nonresponders. Thus the numbers or percentages of Treg cells in asthmatic subjects depend not only on the compartment from which the cells are obtained but also the phenotype of the subjects studied.

Another interesting approach to examine the ability of human Treg cells to attenuate allergen-induced airway responses and inflammation was reported by Martin et al.<sup>23</sup> In this study PBMCs from allergic or healthy donors were injected into NOD/severe combined immunodeficiency mice. This was followed by airway allergen challenge. The investigators activated Treg cells by treatment with the CD4-binding, Ick-activating recombinant HIV-1 surface protein gp120. They concluded that injection of PBMCs from allergic donors, but not healthy donors, allowed the development of allergen-induced airway inflammation and airway hyperresponsiveness and that treatment with gp120 attenuated both responses. By contrast, gp120 had no effect in mice that received CD25-depleted PBMCs. These studies indicate that human Treg cells can attenuate allergen-induced airway responses.

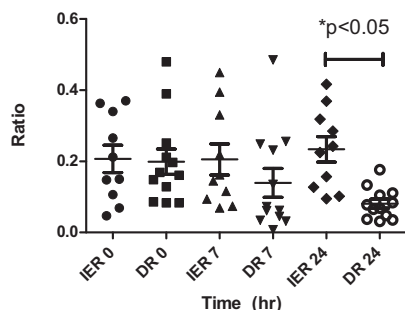
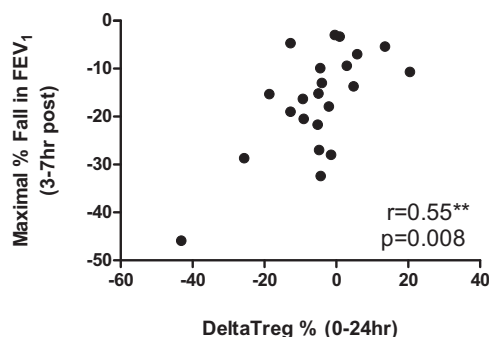
It was because of the differences in the results in studies of asthmatic subjects that we assessed CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell values both in induced sputum and peripheral blood and in allergic asthmatic subjects who had isolated early or dual

responses. We hypothesized those DRs, who have more allergen-induced airway inflammation,<sup>24,25</sup> might have an attenuated Treg cell response associated with enhanced inflammation and prolonged bronchoconstriction. The study demonstrated that both the percentage and absolute number of sputum CD4<sup>+</sup> cells increased whereas the percentage and absolute number of sputum CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells decreased in DRs at 24 hours after inhaled allergen. In addition, the ratio of allergen-induced changes in sputum CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells to CD4<sup>+</sup> cells was significantly lower in DRs when compared with that in IERs. This suggests that the balance between effector T cells and Treg cells might be important in the prolonged airway responses to inhaled allergens. However, an important caveat is that the results do not identify whether the reduced ratio in DRs is a cause or an effect of the airway inflammatory response. The correlation identified between the percentage of sputum Treg cells and the magnitude of the allergen-induced LAR is consistent with both explanations. None of the allergen-induced changes in CD4<sup>+</sup> cells or CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells were demonstrated in peripheral blood. This emphasizes the importance of examining airway cells to understand immune responses after inhaled allergen.

There are a number of limitations to the study. Somewhat surprisingly, although allergen inhalation increased sputum eosinophil numbers, we did not demonstrate significant differences between IERs and DRs. This is different than other studies that we have conducted using similar methodology, in which sputum eosinophil numbers are higher at 24 hours after allergen in DRs.<sup>24,25</sup> The definition of an LAR as a 15% decrease in FEV<sub>1</sub> between 3 and 7 hours after allergen is arbitrary, although widely used. In the current study 5 of the 10 IERs had a decrease in FEV<sub>1</sub>

**TABLE III.** Changes in peripheral blood CD4<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell absolute numbers and percentages after allergen challenge

| Cell type                                                           | Group | Baseline       | 7 h after allergen | 24 h after allergen |
|---------------------------------------------------------------------|-------|----------------|--------------------|---------------------|
| CD4 <sup>+</sup> , number (×10 <sup>3</sup> /mL)                    | DR    | 866.4 ± 182.0  | 1204 ± 413.2       | 1201 ± 343.4        |
| CD4 <sup>+</sup> , % (of CD3 <sup>+</sup> )                         | DR    | 54.11 ± 3.604  | 55.41 ± 2.808      | 52.46 ± 5.150       |
| CD4 <sup>+</sup> Foxp3 <sup>+</sup> , number (×10 <sup>3</sup> /mL) | DR    | 52.81 ± 21.76  | 93.36 ± 52.95      | 86.48 ± 40.22       |
| CD4 <sup>+</sup> Foxp3 <sup>+</sup> , % (of CD4 <sup>+</sup> )      | DR    | 5.005 ± 0.8439 | 5.700 ± 1.043      | 5.476 ± 1.040       |

**FIG 5.** Ratio of absolute Treg cell to CD4<sup>+</sup> cell numbers in sputum of IERs versus DRs.**FIG 6.** Correlation of change in sputum CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell percentages (0-24 hours) versus LARs.

of between 9% and 14%, but because of the *a priori* definition used in designing the protocol, they were included in the early responder group. The measurements of Treg cell numbers in sputum might not reflect the trafficking of these cells within the airway wall; however, the invasive nature of the methods needed to obtain airway tissue makes it very difficult to follow the temporal events occurring after allergen inhalation. Finally, measuring the absolute number of Treg cells in sputum is challenging because of the low frequency of cells. To do this, we used both data from the flow measurements and the absolute sputum cell counts measured with a hemocytometer, which might be less precise than the calculations used to quantify other sputum cells.

In summary, these results demonstrated that CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells can be measured in induced sputum and that this method can be used to follow the time course of events after allergen inhalation, which is not true for measurements in peripheral blood. Allergen inhalation increases CD4<sup>+</sup> cell numbers in DRs, but the ratio of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells as a percentage of CD4<sup>+</sup> cells decreases in DRs when compared with that in IERs. The changes in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell values

correlate with the magnitude of the late response. The results are consistent with the hypothesis that an inability to mobilize sufficient Treg cells into the airways in relation to the increased numbers of effector T cells might be responsible for the prolonged allergen-induced airway responses in DRs.

#### Key messages

- This study demonstrated that CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell numbers can be measured in induced sputum and that this method can be used to follow the changes in Treg cell numbers over time after allergen inhalation.
- The ratio of Treg cell to CD4<sup>+</sup> cell numbers in induced sputum after inhaled allergen is lower in DRs, and the changes in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell numbers correlate with the magnitude of the allergen-induced late response.
- The results suggest that an imbalance in the numbers of inhibitory to effector T cells is important in determining the development of allergen-induced late responses.

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