Mono-allergic and poly-allergic rhinitis patients have comparable numbers of mucosal Foxp3⁺CD4⁺ T lymphocytes*

Abstract
Background: We previously found that allergic rhinitis patients with an isolated pollen sensitization responded more strongly to a nasal provocation with grass pollen (GP) than patients who had an additional house dust mite (HDM) sensitization. To elucidate this phenomenon, we investigated the dynamics of Foxp3⁺CD4⁺ T lymphocytes in allergic rhinitis patients with distinct allergen sensitizations.

Methods: Three groups of allergic rhinitis patients with skin prick test confirmed allergic sensitizations were investigated and compared to 14 healthy controls: 14 subjects with an isolated grass pollen sensitization (Mono-GP); 9 subjects with isolated house dust mite sensitization (Mono-HDM); 29 subjects with grass pollen and house dust mite sensitization (poly-sensitized). Subjects in the Mono-GP group were challenged with grass pollen extract, subjects in the Mono-HDM group were challenged with house dust mite extract, subjects in the poly-sensitized group and the healthy controls were randomly challenged with either grass pollen or house dust mite. Nasal biopsies were taken before and after nasal provocation. We compared the distribution of FoxP3⁺CD4⁺ cells in nasal biopsies before and after nasal provocation using immunohistochemistry.

Results: There was no difference in the number of FoxP3⁺CD4⁺ cells between healthy and the three allergic groups at baseline. Nasal provocation did result in an increase in eosinophils in the three allergic groups, but did not result in a change in the number of FoxP3⁺CD4⁺ cells in any of the groups or induced differences between any of the groups.

Conclusion: Clinical differences in the response between mono-GP and multiple-sensitized allergic individuals are not related to differences in the number of regulatory T cells in the nasal mucosa.

Key words: allergic rhinitis, FoxP3, grass pollen allergy, provocation, regulatory T cells

Introduction
The ARIA guidelines for classification and treatment of allergic rhinitis are based on the severity and duration of clinical symptoms, with more stringent treatments at increasing severities (1). ARIA does not differentiate between different kinds of allergic sensitizations. Nevertheless, two of our clinical studies showed a correlation between sensitization type and level of symptoms. Allergic rhinitis patients with an isolated pollen sensitization responded more strongly to a nasal provocation with grass pollen than patients who had an additional house dust mite (HDM) sensitization (2,3). Interestingly, this observation seems specific for isolated grass pollen allergic individuals as we could not detect any differences after nasal provocation with HDM extract between allergic rhinitis patients with an isolated...
HDM sensitization and those with an additional pollen sensitization \(^{(3)}\).

In the search for the molecular mechanism behind these clinical observations we considered different options. We first investigated the level of allergen-specific immunoglobulins, but neither levels of IgG4 or IgE, nor the biological activities of the immunoglobulins could explain the clinical observations \(^{(3)}\). A clue towards the possible molecular mechanism came when we compared the distribution and dynamics of dendritic cells (DC) in allergic rhinitis patients with different sensitizations. Amongst other things, we observed that after nasal provocation the ratio of myeloid and plasmacytoid DCs (mDC/pDC) increases more strongly in mono-sensitized subjects than in multi-sensitized subjects \(^{(3)}\). Given the postulated role of mDCs in the activation and pDCs in the inhibition of immune responses \(^{(15-17)}\), we suggested that this difference in dendritic cell subset distribution could point to differences in immune suppressive mechanisms. Allergic experimental models in mouse and immune therapy studies in man have linked the expression of the transcription factor forkhead box protein P3 (Foxp3) to a specialized class of regulatory T lymphocytes (T-reg) \(^{(18,19)}\). Direct cell-cell contact and the action of secreted mediators like IL-10 and TGF-β by T-regs have been shown to inhibit immune responses in both pro-inflammatory DCs and T cells \(^{(10-14)}\). Here, we wanted to corroborate and extend our previous clinical and cellular observations at the T-reg level by investigating the level and dynamics of Foxp3+CD4+ T lymphocytes in the nasal mucosa of allergic rhinitis patients with distinct allergen sensitizations.

### Materials and methods

#### Subjects

The allergic subjects in this study suffered from allergic rhinitis for at least two years, while the healthy controls had no medical history pointing to any allergic disease \(^{(3)}\). Subjects with asthma were allowed to participate in the study if they used less than 1000 µg of inhaled corticosteroids per day. All subjects were non-smokers and they had no recent history of upper or lower respiratory tract infection, acute asthma attacks, or hospital admissions during the four weeks prior to investigation. Subjects were excluded if they suffered from a disorder likely to interfere with the test results. All medication for AR treatment was stopped before enrolment: intranasal steroids for one month and anti-histamines for two days. At screening, all subjects were tested for a panel of common inhalant allergens (Alder, Alternaria, Aspergillus fumigatus, Birch, Dermatophagoides pteronysinus and Faria, Cat, Dog, Grass-mix, Hazel. ALK-Abello BV, Nieuwegein, the Netherlands). A skin prick test was considered positive when the wheal diameter was 3 mm larger than that produced by the negative control after 15 minutes. The allergic subjects had a positive skin prick test to grass pollen and/or house dust mite, while the healthy controls had a negative skin prick test.

### Study design

The following distinct groups were formed: subjects with AR and isolated grass pollen sensitization confirmed by skin prick test (Mono-GP, \(n = 14\)); subjects with AR and sensitization to house dust mite without pollen sensitization (Mono-HDM, \(n = 9\)); subjects with AR with increasing symptoms during the pollen season and multiple sensitizations at the skin prick test, including house dust mite and grass pollen (Poly-sens, \(n = 29\)); healthy controls (Controls, \(n = 14\)). In total, 66 eligible subjects (46 female, median age 23.8 yrs; range 18-62 yrs) were included in this study. Patient characteristics are shown in Table 1.

Subjects in the Mono-GP group were challenged with grass pollen extract (grasses mix, ALK-Abello BV), subjects in the Mono-HDM group were challenged with house dust mite extract (Dermatophagoides pteronysinus, ALK-Abello BV). Subjects in the poly-sensitized group and the healthy controls were randomly challenged with either grass pollen or house dust mite allergen. Subjects were challenged with 10,000 BU/mL grass pollen or house dust mite extract (ALK-Abello) via a pump spray delivering one fixed dose of 89 µL into each nostril.

The study was conducted outside the grass pollen season and all participants gave written informed consent to the study, which was approved by the medical ethics committee of the Academic Medical Center (MEC 03/201), Amsterdam, the Netherlands.

#### Nasal biopsies and immunohistochemical staining for FoxP3 and CD4

Nasal biopsies were taken before and 24 hours after nasal allergen provocation. All biopsies were taken by the same investigator (S.M.R.). Local anesthesia was induced by placing a cotton-wool carrier with 50-100 mg of cocaine and three drops of epinephrine (1: 100,000) under the inferior turbinate, without touching the biopsy site. Then, a mucosal biopsy sample was taken before enrolment: intranasal steroids for one month and anti-histamines for two days. At screening, all subjects were tested for a panel of common inhalant allergens (Alder, Alternaria, Aspergillus fumigatus, Birch, Dermatophagoides pteronysinus and Faria, Cat, Dog, Grass-mix, Hazel. ALK-Abello BV, Nieuwegein, the Netherlands). A skin prick test was considered positive when the wheal diameter was 3 mm larger than that produced by the negative control after 15 minutes. The allergic subjects had a positive skin prick test to grass pollen and/or house dust mite, while the healthy controls had a negative skin prick test.

### Table I. Subject characteristics.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years) Median (range)</th>
<th>Gender (% Female)</th>
<th>Der.p-IgE (kU/L) Median (range)</th>
<th>P.prat-IgE (kU/L) Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-HDM (14)</td>
<td>22 (19-62)</td>
<td>44</td>
<td>10.6 (0.6 - 43.6)</td>
<td>0 (0 - 0.6)</td>
</tr>
<tr>
<td>Mono-GP (9)</td>
<td>28 (18-53)</td>
<td>57</td>
<td>0.1 (0 - 0.2)</td>
<td>9.7 (1.2 - 92.6)</td>
</tr>
<tr>
<td>Polysens-provHDM (14)</td>
<td>23 (18-59)</td>
<td>77</td>
<td>12.1 (0.1 - 306)</td>
<td>9.2 (0.1 - 318)</td>
</tr>
<tr>
<td>Polysens-provGP (15)</td>
<td>22 (18-39)</td>
<td>56</td>
<td>25.9 (0.1 - 300)</td>
<td>8.7 (0.1 - 148)</td>
</tr>
<tr>
<td>Healthy controls (14)</td>
<td>22 (19-52)</td>
<td>71</td>
<td>0 (0 - 1.0)</td>
<td>0 (0 - 0)</td>
</tr>
</tbody>
</table>
obtained from the lower edge of the inferior turbinate about 2 cm posterior to the edge, using a Fokkens forceps with a cup diameter of 2.5 mm. Nasal biopsies were embedded in Tissue-Tek II Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA, USA), frozen, cut into serial 5-µm-thick sections, transferred onto slides, dried, and stored at -80°C until use.

Before staining, slides were heated to room temperature and subsequently dried and fixed in acetone for 10 min. Slides were PO-blocked with azide (2%), peroxidase (30%) in PBS phosphate-buffered saline (PBS; pH 7.4). Then rinsed in phosphate-buffered saline (PBS; pH 7.4), and 10% normal goat serum (NGS) in blockbuffer was added for 10 min. Subsequently, slides were incubated with a cocktail mix with rabbit polyclonal antibody to Foxp3 (1:200; Abcam, Cambridge, MA, USA) and mouse monoclonal antibody to CD4 (1:1,000; BD, Franklin Lakes, NJ, USA) for 60 min. Sections were rinsed with PBS, incubated with a cocktail mix with powervision goat anti-mouse-HRP (pure; Immuno vision Technologies, Daly City, CA, USA) and powervision goat anti-rabbit-AP (pure; Immuno vision Technologies) for 30 min, and rinsed with PBS. Next, slides were incubated with 3,3’-Diaminobenzidine substrate (DAB; kit Dako; Glostrup, Denmark). Sections were then rinsed with PBS and rinsed with Tris buffer (0.1 M, pH 8.5), and incubated for 30 min with Fast blue B substrate. The sections were rinsed in PBS and then in distilled water, dried overnight at room temperature and mounted in Vectamount. Control sections, incubated with irrelevant mAbs of the same subclass and at the same protein concentration as the specific antibody, were negative.

Statistical analysis
We compared the distribution of different cell types from independent groups (different disease modalities) using the Kruskal-Wallis test or between related samples (before and after nasal allergen provocation) with the Friedman test. A p-value of less than 0.05 was considered significant.

Results
Presence of regulatory T cells in nasal mucosa
By using double staining, we were able to identify varying absolute numbers of FoxP3 CD4 double positive cells within the lamina propria at baseline (Figure 1). We found no statistical significant differences between healthy and allergic individuals or between mono and multiple sensitized allergic rhinitis patients (Figure 2A). Median numbers in healthy individuals (12.3 cells/mm² LP) ranged from 2.1 to 27.1 cells/mm² LP, while for mono-HDM, Mono-GP, and multiple sensitized individuals these numbers were 11.4 cells/mm² LP (range 0 – 55.6), 7.6 cells/mm² LP (range 0 – 23.6), and 11.9 cells/mm² LP (range 0 – 77.4) respectively. As all FoxP3+ cells were also CD4+ we also determined the fraction of FoxP3+CD4+ cells as a percentage of total CD4+ cells. With varying absolute numbers of total CD4+ cells, and no differences between groups (Figure 2B, p = 0.451), also the relative number of FoxP3+CD4+ cells did not differ between groups (p = 0.334, Figure 2C). The median percentages in healthy individuals were 1.96% (range 0.33 - 3.52%), while for mono-HDM, Mono-GP, and multiple sensitized individuals these numbers were 1.68% (range 0 - 3.82), 1.57 (range 0 – 3.66%), and
FoxP3CD4 expression in allergic nasal mucosa

1.71% (range 0 – 13.9%), respectively.

Nasal provocation does not influence number of regulatory T cells
Next, we investigated the dynamics of FoxP3+CD4+ cells after nasal provocation. The mono-sensitized patients just received provocation with the relevant allergen. In the healthy control group and the multiple-sensitized group, half of the group received a nasal provocation with grass pollen and the other half a provocation with house dust mite extract. Nasal provocation did induce eosinophilia in all allergic groups (Figure 3A, p < 0.001) with the number of eosinophils changing from median 0.52 (range 0 – 12.56) to 7.22 (range 0 – 27.6) for mono-GP, from median 2.65 (range 0 – 53.3) to 8.38 (range 0.19 – 32.4) for mono-HDM, from median 0.55 (range 0 – 11.97) to 7.50 (range 0.12 – 21.96) for poly-sensitized after GP provocation, and from median 1.77 (range 0 – 8.27) to 6.87 (range 0 – 33.08) for poly-sensitized after HDM provocation, while the number of eosinophils in healthy individuals did not change.

However, despite these changes in the number of eosinophils, the nasal provocation did not induce a discernible change in number of CD4 cells (Figure 3B, p = 0.211) for all groups or the absolute (Figure 3C, p = 0.597) or relative (Figure 3D, p = 0.575) number of FoxP3+CD4+ cells. As a consequence also after nasal allergen provocation no differences between groups could be detected.

Discussion
We could not show any differences between absolute or relative numbers of FoxP3+CD4+ cells in nasal mucosa depending on the diseased state. Numbers were not different between healthy and allergic individuals; neither did they differ depending on the kind or multitude of the allergic sensitization, nor as a measure of the activation of the immune response after nasal provocation.

Previously, we have shown that patients with a concomitant HDM sensitization responded less strongly to a grass pollen provocation than patients with an isolated pollen sensitization (8). The changed ratio of pDC versus mDC (9) and the role of pDCs (10-12) in suppressing immune responses suggested that there could be a role for differential levels of regulatory T cells depending on the sensitization pattern. FoxP3 has been described as the hallmark of regulatory cells. Indeed in mouse models the argument is strong (13-15) and successful immunotherapy in man has been linked to the appearance of FoxP3+CD4+ cells (14,16). However, no data was available to show a possible role for FoxP3+CD4+ cells controlling symptoms during normal or induced allergen exposures. The regulatory function has been extended when it was shown that not only CD4+ cells but also CD8+ cells can express FoxP3 (17,18). Recently, even an innate type of lymphocyte...
Figure 3A. Absolute number of eosinophils per mm² lamina propria of nasal biopsies both before (light grey) and after (dark grey) provocation. Different groups were investigated: healthy controls and allergic rhinitis patients with different types of allergic sensitizations (Monosens HDM = isolated HDM AR, Monosens GP = isolated grass pollen AR) and allergic rhinitis patients with increasing symptoms during the pollen season and multiple sensitizations (Polysens), exposed to HDM (provHDM) or to grass pollen (provGP).

Figure 3B. Absolute number of CD4 positive cells per mm² lamina propria of nasal biopsies both before (light grey) and after (dark grey) provocation. Allergen provocation did not induce any significant changes in healthy controls and allergic rhinitis patients with different types of allergic sensitizations (Monosens HDM = isolated HDM AR, Monosens GP = isolated grass pollen AR) and allergic rhinitis patients with increasing symptoms during the pollen season and multiple sensitizations (Polysens), exposed to HDM (provHDM) or to grass pollen (provGP).

Figure 3C. Absolute number of FoxP3-CD4 double positive cells per mm² lamina propria of nasal biopsies both before (light grey) and after (dark grey) provocation. Allergen provocation did not induce any significant changes in the number of FoxP3+CD4+ cells in healthy controls and allergic rhinitis patients with different types of allergic sensitizations: (Monosens HDM = isolated HDM AR, Monosens GP = isolated grass pollen AR) and allergic rhinitis patients with increasing symptoms during the pollen season and multiple sensitizations (Polysens), exposed to HDM (provHDM) or to grass pollen (provGP).

Figure 3D. Fraction (%) of FoxP3+CD4+ double positive cells of the total amount of CD4 positive cells in the lamina propria of nasal biopsies both before (light grey) and after (dark grey) provocation were similar for healthy controls and allergic rhinitis patients with different types of allergic sensitizations (Monosens HDM = isolated HDM AR, Monosens GP = isolated grass pollen AR) and allergic rhinitis patients with increasing symptoms during the pollen season and multiple sensitizations (Polysens), exposed to HDM (provHDM) or to grass pollen (provGP).
has been identified that expresses FoxP3 [21,22] albeit that for this particular cell type the function is still unclear. If we would indeed consider the FoxP3+CD4+ cells to represent regulatory T cells it would seem that the differences in the clinical response between mono-GP allergic individuals and those with a concomitant HDM sensitization is not regulated at the level of their cell numbers. This would still leave the possibility that the activity of regulatory T cells is affected [23,24]. However, currently is it unclear what should be used as an in vivo marker to define T-reg activity in immunohistochemistry, where unlike FACS-based analyses, we would not have the ability to discern different levels of expression. In this regard, our avenue of taking biopsies after in vivo exposure does not allow us to address this option. In general, immune regulation and the role of T cells and/or dendritic cells is often considered only in the context of the start or the termination of the immune response and not in the effector phase. However, this might not be true as elegant mouse experiments have shown that ablation of DCs after allergy has been established, will prevent the induction of allergic symptoms after allergen provocation [25]. Evidently loading and/or cross-linking of IgE on mast cells is not sufficient to induce clinical symptoms in vivo, despite that in vitro DCs are not required for the activation of mast cells. Now that we have failed to show an involvement of FoxP3+CD4+ cells in the differences of the responsiveness of allergic individuals it might still be that differences we reported at the dendritic cell level [4], with a stronger increase in the mDC/pDC level in mono-GP allergic individuals, might instead have a direct bearing on effector cells, rather than acting through T cell subsets. Whether this mechanism would underlie our clinical observations needs to be further explored. An alternative explanation that the differences on the DC level would act through a differential activation of T helper cells seems unlikely. The clinical differences can be observed in the early allergic response, as little as 15 minutes after nasal provocation. This would probably still require a modulating effect on mast cells, as adaptive T cells (memory, regulatory, or effector) have not been described in this light. Whether the newly described class of innate T cells [25,26] could play a role remains unexplored. As innate responses are generally seen as quick responses to potential threats, this avenue of research could be a valid option. Alternatively, it could be explored if local allergen-specific IgE responses might differ, despite an absence of differences in systemic allergen-specific IgE responses [27]. Although FoxP3 is expressed in regulatory CD4 T cells in man there is sufficient data to suggest that also effector T cells express FoxP3 [27-29]. In man, FoxP3 is transiently up-regulated as part of the immune response, albeit that in FACS-based analysis for FoxP3 the expression in activated T cells is described as dim, whereas regulatory cells stably express FoxP3 at a higher level. Given that immunohistochemical analysis does not necessarily show this same distinction of brightly and dimly staining cells, we cannot be sure if the FoxP3+CD4+ cells we have enumerated, just reflect the FoxP3 HIGH regulatory cells or also the FoxP3 DIM activated T cells. In the mono-GP sensitized individuals the potential issue of activated T cells expressing FoxP3 is unlikely to play a role, as patients were seen outside the pollen season, where no pollen-related activated T cells will be expected. In case of a concomitant HDM sensitization the problem remains, as individuals are constantly exposed to HDM in everyday life, even when clinical symptoms may only clearly induced under specific conditions of increased exposure at home or in a clinical setting [30]. In such a chronic context, the FoxP3+CD4+ T cells might represent both a regulatory as well as an activated phenotype, however, we could not detect any differences between any of the allergic groups in FoxP3 positive T cells at baseline. Moreover, the number of FoxP3 positive T cells in the allergic groups did also not differ from the number of FoxP3 positive T cells in healthy controls. This is in line with the observations of Radulovic and co-workers who also could not detect any differences in tissue numbers of FoxP3 positive T cells between a pollen immunotherapy group at baseline and healthy controls [31]. We cannot exclude the possibility that in a bigger cohort of patients some of the differences in cell numbers between before and after HDM provocation in the mixed rhinitis group might reach statistical significance. However, this would fail to explain the high symptom load in mono-GP allergic individual, with overall the differences between median cell numbers in different clinical groups are in the order of 10%. The observation of differences in the number FoxP3 positive T cells that depend on the allergic status may well depend on what compartment of the immune system is sampled. In contrast to our observations and those of Radulovic and co-workers in tissue, other reports show that T cells isolated from blood or lung lavages is decreased in allergic individuals in either the number of T cells expressing FoxP3 or the ability of naive T cells to acquire a FoxP3 positive phenotype [31,32].

Conclusion
Summarizing, our data suggest that the clinical differences in the response between mono-GP and multiple-sensitized allergic individuals (and the differences in the mDC/pDC ratio’s) are not related to differences in the number of tissue resident FoxP3 positive T cells in nasal mucosa at baseline and also not related to a change in their number due to a one-time allergen exposure.

Authorship contribution
BM: Evaluating and interpretation of data, writing part of the manuscript, evaluating and commenting the manuscript, approving the manuscript; SMR: Collecting material, interpretation of data, writing part of the manuscript, evaluating and commenting the manuscript; DvE: measurements of data, evaluating and commenting the manuscript; EJJdG: measurements of the
data, evaluating and commenting the manuscript; WJF: design of study, interpretation of data, evaluating, commenting and approving the manuscript.

Conflict of interest
No conflicts of interest to disclose.

References