

ORIGINAL PAPER

Depletion of CD4⁺CD25⁺CD127^{lo} regulatory T cells does not increase allergen-driven T cell activationI. Skrindo^{*†}, L. Farkas^{*}, E. O. Kvale^{*}, F.-E. Johansen^{*‡} and F. L. Jahnsen^{*‡}^{*}Laboratory for Immunohistochemistry and Immunopathology, Centre for Immune Regulation, Institute of Pathology, University of Oslo, Oslo, Norway, [†]ENT Department, Rikshospitalet, Oslo, Norway and [‡]Division of Pathology, Rikshospitalet, Oslo, NorwayClinical and
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Summary

Background It has been suggested that allergic diseases are caused by defective suppression of allergen-specific Th2 cells by CD4⁺CD25⁺ regulatory T cells. However, such studies have been hampered by the difficulty in distinguishing regulatory T cells from CD25-expressing activated T cells. Recently, it was shown that conventional T cells expressed high levels of CD127, whereas regulatory T cells were CD127^{lo}, allowing discrimination between these distinct T cell subpopulations.

Objective The aim of this study was to study whether the putative regulatory subset defined as CD4⁺CD25⁺CD127^{lo} was involved in grass pollen-reactive T cell responses.

Methods Peripheral blood mononuclear cells (PBMCs) were obtained from allergic donors and non-atopic controls out of season. Grass pollen-induced cytokine production and proliferation were compared in cultures of undepleted cells and cells depleted of CD4⁺CD25⁺, CD4⁺CD25⁺CD127^{hi} or CD4⁺CD25⁺CD127^{lo} T cells.

Results Undepleted cell cultures from allergic patients showed significantly increased proliferation and Th2 cytokine production compared with non-atopic controls. Depletion of all CD25⁺ T cells did not increase cytokine production or proliferation, and more importantly, no increase in Th2 cytokine production or proliferation was observed in cell cultures depleted of CD4⁺CD25⁺CD127^{lo} cells (putative regulatory T cells) compared with undepleted PBMCs in both the allergic and the non-atopic group.

Conclusion Our study showed that T cells from grass pollen-allergic patients and non-atopic controls responded very differently to grass pollen extract, but this difference could not be explained by differences in regulatory T cell function. Further studies are needed to understand the importance of regulatory T cells in allergy.

Keywords allergic rhinitis, allergy, human, regulatory T cells, T cells, Th2 cytokines

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Introduction

A dysregulated T cell response to allergens is central to the pathogenesis of allergic reactions. This response is dominated by CD4⁺ T helper 2 cells that produce cytokines such as IL-4, IL-5 and IL-13. IL-4 and IL-13 are crucial switch factors for IgE production, whereas IL-5 is essential for eosinophil activation, both hallmarks of the allergic reaction [1–3]. In contrast to a Th2-dominated response to allergens in allergic individuals, T cells isolated from peripheral blood of healthy, non-atopic individuals typically show a low-level Th1 response [4–6].

A question that is still unresolved is why some individuals develop inflammatory reactions to harmless antigens, whereas others handle the same antigens without signs of inflammation. The dogma of the 1990s that allergic reactions result from an imbalance between Th1 and Th2 responses has been challenged by recent information suggesting that so-called regulatory T (Treg) cells are important regulators of both Th1 and Th2 responses. Treg cells, defined as CD4⁺CD25⁺Foxp3⁺ T cells, can be divided into two main subgroups: a naturally occurring thymus-derived subset, and an adaptive subset, which appears to differentiate from CD4⁺CD25⁺ memory T cells in the periphery [1, 7].

In a recent study by Ling et al. [8], it was shown that CD25-depleted CD4⁺ T cells isolated from grass pollen-allergic and non-atopic donors produced similar amounts of Th2 cytokines in response to grass pollen extract. However, addition of autologous CD4⁺CD25⁺ T cells completely abrogated this Th2 response, but only in the non-atopic group. These findings suggested that upper airway allergy is caused by defective suppression of allergen-specific Th2 cells by CD4⁺CD25⁺ Treg cells. However, more recently, other groups have performed similar studies with rather disparate results [9–11], which question the role of Treg cells in upper airway allergy. A common problem that complicates the interpretation of these studies is that they define Treg cells as CD4⁺CD25⁺ peripheral blood T cells. However, the expression of CD25 does not allow distinction between Treg cells and activated effector T cells. Indeed, as shown by Grindebacke et al. [9], isolated CD4⁺CD25⁺ T cells stimulated with pollen extracts produced similar levels of Th2 cytokines compared with the CD4⁺CD25[−] T cell population during the birch pollen season, suggesting that a substantial fraction of the CD4⁺CD25⁺ T cell population was allergen-reactive effector T cells.

To discriminate between CD25⁺ Tregs- and CD25⁺-activated effector T cells, we have taken advantage of recent information, which showed that activated conventional T cells express high levels of CD127, whereas Treg cells are CD127^{lo} [12, 13]. In this study, we compared grass pollen-specific T cell responses in T cell subsets defined by their expression of CD4, CD25 and CD127.

Materials and methods

Participants

Medical students and laboratory staff were recruited for this study. Venous blood was obtained from 17 randomly selected allergic donors, median age 26 years (range 21–57, eight women), and 13 non-atopic donors, median age 24 years (range 22–28, 10 women), outside the grass pollen season. They were all skin prick tested with a panel of eight common inhalant allergens (house dust mite, mould, horse, dog, cat, grass, mugwort and birch) (Solu Prick SQ, ALK-Abello A/S, Horsholm, Denmark). The allergic volunteers had a typical history of grass pollen-induced upper airway allergy and a positive skin prick test to grass pollen according to the European guidelines [14]. Of these, 9 out of 17 also had a positive skin prick test to one or more allergens in addition to grass. All non-atopic volunteers had a history free of allergic symptoms and a negative skin prick test to the same allergen panel. For suppression assays, venous blood was obtained from three healthy adults without knowledge of their atopic status (23–37 years old). Peripheral blood mononuclear cells (PBMCs) from one of the donors were used as responder

cells and antigen-presenting cells (APCs) in two independent experiments.

The study was approved by the Norwegian Regional Committee for Medical Research Ethics, and written consent was obtained from all participating subjects.

Cell isolation and culture

PBMCs were obtained by Lymphoprep gradient centrifugation (Axis-Shield PoC, Oslo, Norway). Depletion of cells was either performed with CD25 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's manual or by cell sorting. Briefly, isolated PBMCs were incubated with CD25 MicroBeads for 15 min at 4 °C, washed and run through an LD column. In cell-sorting experiments, isolated PBMCs were incubated for 30 min at 4 °C with the following monoclonal antibodies: CD127PE, CD25PECy5 and CD4APC or CD127PE, CD25APC and CD4FITC (BD Biosciences, San Diego, CA, USA), and three different subpopulations of cells were depleted (CD4⁺CD25⁺, CD4⁺CD25⁺CD127^{lo} and CD4⁺CD25⁺CD127^{hi}) using a FACS Aria or FACS DiVa (both BD Biosciences). Identically immunostained PBMCs that had passed through the sorter without any depletion were used as controls.

PBMCs left undepleted or depleted for different T cell subpopulations were washed and resuspended at a concentration of 2×10^6 cells/mL. A total of 2×10^5 cells were then cultured in RPMI containing 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (all from Camprex Bio Science Verviers, Verviers, Belgium) supplemented with 10% human pooled and heat-inactivated serum, with or without *Phleum pretense* (Phl p, timothy grass pollen extract, 20 µg/mL; ALK-Abello) in triplicate or quadruplicate in u-bottom 96 well plates (Costar Corning, Corning, NY, USA) for 7 days at 37 °C.

Supernatants were collected after 4 h (in some experiments) and on day 6 and frozen at −70 °C for later measurement. At day 6, 100 µL medium (as described earlier) containing 1 µCi/well [3H]-thymidine was added. [3H]-Thymidine incorporation was measured after 16 h. Thymidine incorporation is shown as counts per minute (cpm) or as a proliferative index (PI; proliferation in stimulated cultures divided by proliferation in unstimulated cultures).

Suppression assay

Suppression assays were performed as described previously [12]. Briefly, 1×10^5 responder PBMCs were cultured for 6 days together with sorted autologous CD4⁺CD25⁺CD127^{lo} T cells at concentrations as indicated. A ratio of 1:1 refers to 30 000 sorted cells: 100 000 PBMC responder cells. A total of 1×10^5 allogeneic anti-CD3-depleted, irradiated PBMCs were used as APCs.

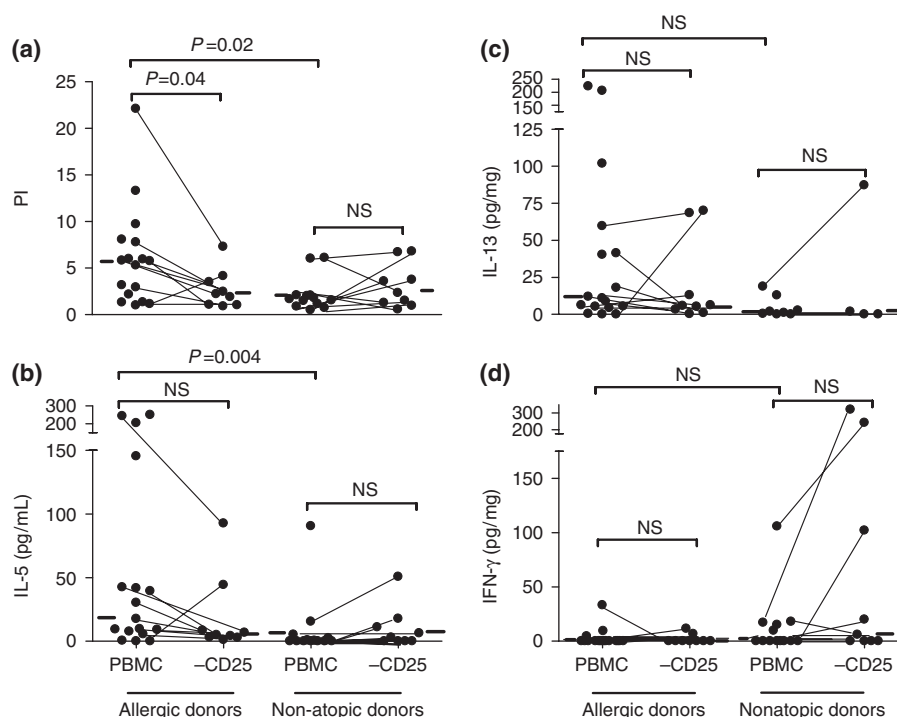


Fig. 1. Peripheral blood mononuclear cells (PBMCs) obtained from allergic ($n = 17$) and non-atopic ($n = 13$) donors were cultured with and without allergen (Phl p) for 7 days. In some experiments ($n = 9$ for allergic patients and non-atopic individuals, respectively), PBMC cultures depleted of CD25⁺ cells with MACS beads were compared with untouched PBMCs (indicated by lines). The two-tailed Wilcoxon signed rank test and the Mann-Whitney *U*-test were performed for paired and unpaired statistical analysis, respectively: (a) proliferative index measured by thymidine incorporation; (b–d) cytokine measurements. Horizontal bars indicate medians. NS, non-significant.

CD3 depletion was performed with CD3 Dynabeads (DynaL Biotech ASA, Oslo, Norway) according to the manufacturer's manual. Proliferation was measured as described above.

Cytokine measurements

Cytokine measurements were performed either with a BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit and analysed with a FACS Calibur (all from BD Biosciences), or with a Bio-Plex Human Cytokine 27-Plex Panel from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) analysed with a Luminex machine (Luminex xMAP Technology from Bio-Rad Laboratories). The results were given as values in stimulated cultures minus basal levels.

Statistical analysis

The two-tailed Wilcoxon signed rank test and the Mann-Whitney *U*-test were performed to compare the differences within one group or differences between allergic patients and controls, respectively. The statistical tests were performed with Graph pad Prism 4.0. A *P*-value of <0.05 was considered significant.

Results

Grass pollen-stimulated peripheral blood mononuclear cell cultures from allergic individuals proliferate better and produce more interleukin-5 than their non-atopic counterparts

To examine the grass pollen-dependent T cell response in allergic and non-atopic individuals, we measured the proliferation and cytokine production in undepleted PBMC cultures (Fig. 1). As expected, we found that PBMCs obtained from allergic individuals proliferated significantly better ($P = 0.02$; Fig. 1a) and produced significantly more IL-5 ($P = 0.003$; Fig. 1b) than PBMCs from the non-atopic group. The levels of IL-13 (Fig. 1c) also tended to be higher in the allergic group ($P = 0.06$), but did not reach statistical significance, whereas IFN- γ levels were similar in both groups (Fig. 1d). In some experiments, we also measured the cytokine levels in the supernatants after 4 h of grass pollen stimulation in undepleted PBMC cultures from allergic patients ($n = 4$) and non-atopic donors ($n = 4$). However, no cytokines could be detected at this early time-point. This finding indicated that IL-5 and IL-13 were not pre-stored in granula and secreted from effector cells such as basophils, but depended on protein synthesis in T cells.

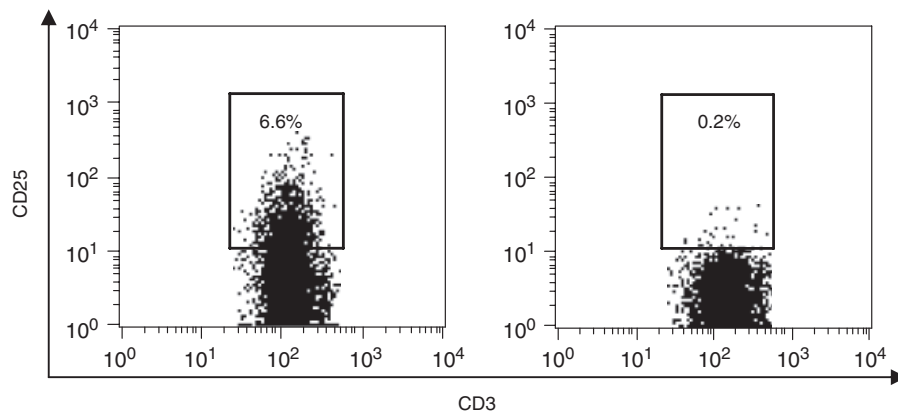


Fig. 2. Flow-cytometric analysis of peripheral blood mononuclear cells before (left panel) and after (right panel) depletion with CD25 MACS beads. Gated region indicates $CD3^+CD25^+$ cells (percentages of $CD3^+$ that are $CD25^+$). The plots are representative of 18 independent experiments performed.

Magnetic-bead depletion of $CD25^+$ T cells does not increase grass pollen-dependent proliferation or cytokine production

In a subset of these individuals, we also examined the effect of $CD4^+CD25^+$ Treg cells on grass pollen-dependent T cell responses by magnetic-bead depletion of $CD25^+$ T cells before culture. We found that a median of 3.8% (range 1.4–6.6%, nine allergic and nine non-atopic donors) of $CD3^+$ T cells expressed CD25. After depletion, this fraction was reduced to 0.5% (range 0.1–1.6%) (Fig. 2). The fraction of $CD25^+$ T cells was not different in allergic donors compared with non-atopic donors (not shown). Compared with undepleted PBMC cultures, the proliferative response in $CD25$ -depleted PBMCs from the allergic group was significantly reduced ($P=0.04$), whereas the proliferative response was unchanged in the non-atopic group (Fig. 1a). There was no significant difference in the levels of IL-5, IL-13 or IFN- γ between undepleted and $CD25$ -depleted cultures in either of the groups (Figs 1b–d).

Depletion of $CD4^+CD25^+CD127^{lo}$ putative regulatory T cells does not affect grass pollen-dependent proliferation or cytokine production

CD25 is also expressed on conventional activated T cells, and it is therefore possible that allergen-reactive effector T cells were depleted using the magnetic-bead separation procedure. Thus, we performed cell-sorting experiments, which allowed us to separate $CD4^+CD25^+$ Tregs from $CD4^+CD25^+$ T effector cells by their differential expression of CD127 [12, 13]. By this staining strategy, the $CD4^+CD25^+$ population was divided into two distinct subsets: $CD4^+CD25^+CD127^{lo}$ and $CD4^+CD25^+CD127^{hi}$ cells. As shown in Fig. 3, there was no difference in cell numbers within these two subsets between the allergic and the non-atopic group. Moreover, the size of these

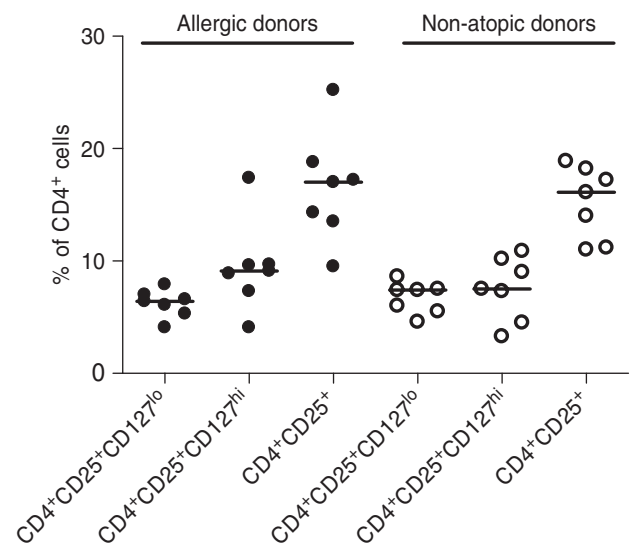


Fig. 3. Percentages of $CD4^+CD25^+CD127^{lo}$, $CD4^+CD25^+CD127^{hi}$ and $CD4^+CD25^+$ T cells in peripheral blood mononuclear cells from allergic and non-atopic individuals. Horizontal bars indicate medians.

cell fractions correlated well with that published by others [12, 13].

The $CD4^+CD25^+CD127^{lo}$ subset has been shown to display suppressive activity [12, 13]. We therefore examined the ability of this putative Treg population to suppress an allogeneic mixed lymphocyte reaction. Indeed, as shown in Fig. 4, the addition of increasing numbers of $CD4^+CD25^+CD127^{lo}$ T cells had an apparent inhibitory effect on T cell proliferation; 15 000–30 000 sorted Treg cells co-cultured with 100 000 autologous responder PBMCs and allogeneic APCs reduced the proportion of proliferating cells by more than 50%.

Then, we examined the suppressive effect of this Treg subpopulation on grass pollen-dependent T cell responses. PBMC cultures were depleted of either $CD4^+CD25^+CD127^{lo}$ (Treg cells), $CD4^+CD25^+CD127^{hi}$ (activated T cells) or all $CD4^+CD25^+$ cells, and the pollen-dependent

proliferative response and production of cytokines in the cell-depleted cultures were compared with undepleted PBMCs. Efficient depletion (always better than 95%) was

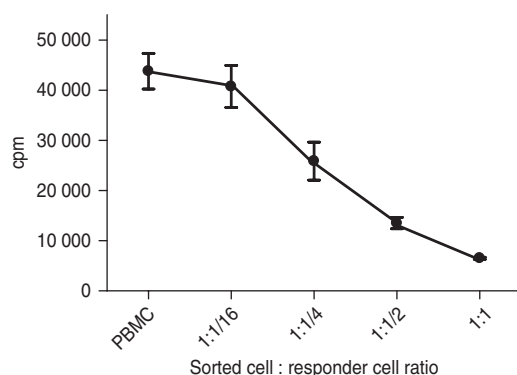


Fig. 4. A total of 100 000 peripheral blood mononuclear cells (PBMCs) were cultured for 7 days without or with increasing numbers of autologous $CD4^+CD25^+CD127^{lo}$ T cells and 100 000 allogeneic anti-CD3-depleted, irradiated PBMCs as antigen-presenting cells. A ratio of 1 : 1 refers to 30 000 sorted $CD4^+CD25^+CD127^{lo}$ T cells: 100 000 PBMC responder cells. The results (mean $\pm$ SD of triplicate wells) are representative of two independent experiments cpm, counts per minute.

verified by flow analysis (Fig. 5). Figure 6 shows that there was no difference in the proliferative response and cytokine production between pollen-stimulated undepleted PBMCs and PBMCs depleted of $CD4^+CD25^+CD127^{lo}$ cells. Also, no significant differences were observed when $CD4^+CD25^+CD127^{hi}$ cells or all $CD4^+CD25^+$ cells were depleted. Importantly, in all situations, cell cultures from non-atopic individuals showed a low proliferative response and low levels of IL-5 and IL-13 production. IL-10 levels were always low in both groups (< 20 pg/mL and in most experiments < 5 pg/mL) and were not influenced by the addition of grass pollen extract or by depletion of the various $CD25^+$ T cell subpopulations.

Discussion

In this study, we showed that PBMCs obtained out of season from grass pollen-allergic individuals proliferated better and produced significantly higher levels of IL-5 than their non-atopic counterparts in response to grass pollen extracts. Importantly, depletion of $CD4^+CD25^+CD127^{lo}$ Treg cells from these cultures had no significant effect on grass pollen-dependent proliferation or cytokine

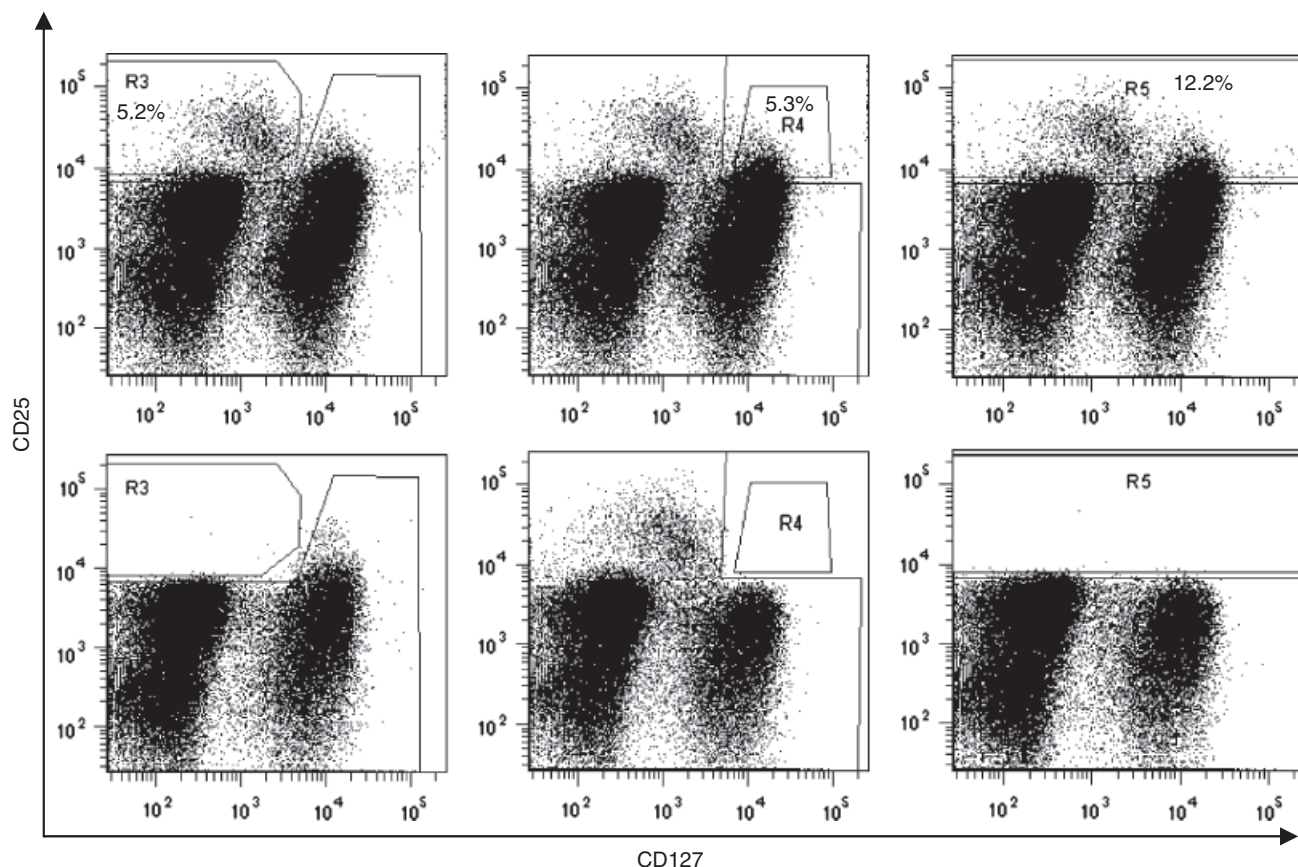


Fig. 5. Expression of CD25 and CD127 in peripheral blood mononuclear cells before (upper panels) and after depletion of $CD4^+CD25^+CD127^{lo}$ cells (R3, left panels), $CD4^+CD25^+CD127^{hi}$ cells (R4, middle panels) and $CD4^+CD25^+$ cells (R5, right panels) by cell sorting. Percentages denote $CD4^+$ cells found in the indicated region. The plots are representative of 14 independent experiments.

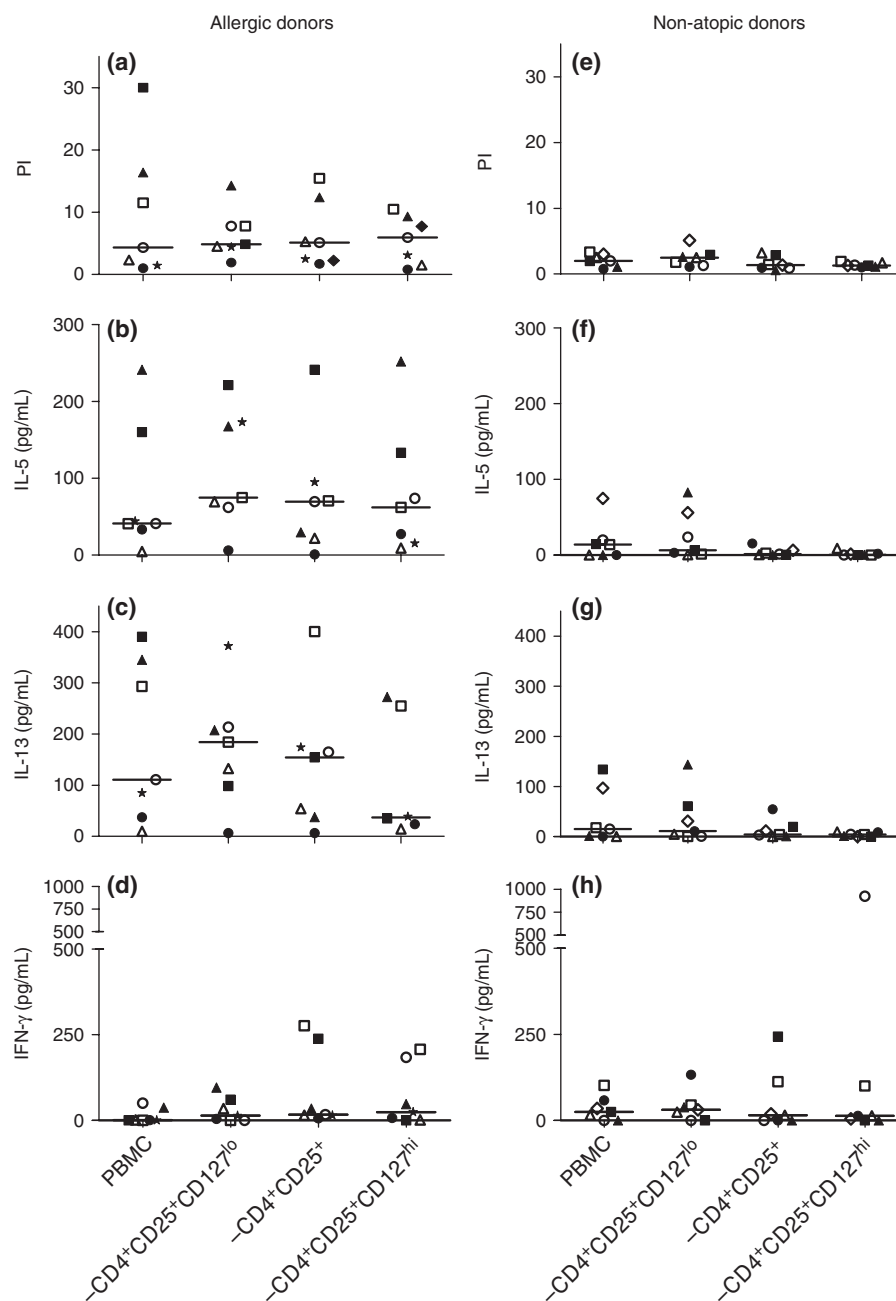


Fig. 6. Peripheral blood mononuclear cells (PBMCs) obtained from allergic ($n=7$) and non-atopic ($n=7$) donors were either depleted of $CD4^{+}CD25^{+}CD127^{lo}$ cells, $CD4^{+}CD25^{+}CD127^{hi}$ cells or $CD4^{+}CD25^{+}$ cells (shown in Fig. 5) and cultured with and without allergen (Phl p) for 7 days. Undepleted PBMCs were stained as the other cells and run through the sorter without cell depletion. Thymidine incorporation is shown as a proliferation index (PI) in (a) (allergic donors) and (e) (non-atopic donors). Cytokine expression is shown in (b–d) (allergic donors) and (f–h) (non-atopic donors). Each individual is indicated by different symbols. Horizontal bars indicate medians.

production in either of the groups. Our finding suggests that this subset of Treg cells does not suppress grass pollen-induced T cell responses at the normal Treg-to-Teffector ratio in peripheral blood when performed outside the season.

Whether $CD4^{+}CD25^{+}$ Treg cells affect T cell-dependent allergen responses has been investigated by several laboratories. Ling et al. [8] showed that $CD4^{+}CD25^{+}$ cells

significantly suppressed grass pollen-dependent IL-5 production, especially in non-atopic individuals out of season. Grindebacke et al. [9] also reported that $CD4^{+}CD25^{+}$ T cells inhibited birch pollen-dependent T cell proliferation and production of Th2 cytokines outside the season, although the effect on non-atopic individuals was rather marginal. Moreover, both studies showed that the suppressive effect by $CD4^{+}CD25^{+}$ T cells was completely

abrogated during the season, but only in the allergic group. However, interpretation of the results in the latter study was complicated by the fact that the putative Treg population obtained from allergic individuals in the season produced high levels of both IL-5 and IL-13, similar to their CD4⁺CD25⁻ counterparts. This finding indicated that the CD4⁺CD25⁺ T cell population had a large number of allergen-specific Th2 effector cells. Similarly, Bellinghausen et al. [15] found that CD4⁺CD25⁺ T cells proliferated equally well and produced similar levels of IL-4 compared with CD4⁺CD25⁻ T cells when incubated with optimal concentrations of wasp venom. In another study examining the effect of CD4⁺CD25⁺ T cells on birch pollen-dependent T cell responses outside the pollen season, Thunberg et al. [10] found that CD4⁺CD25⁺ T cells only suppressed IFN- γ production, but had no effect on the production of Th2 cytokines.

These different results using a rather similar methodology are not readily explained. A major problem, however, in all these studies is their use of CD25 as a marker for isolation of Treg cells. CD25 does not discriminate between regulatory and activated T cells, and as shown by Grindebacke et al. [9], the CD4⁺CD25⁺ T cell population may contain significant numbers of allergen-specific effector T cells, especially during the pollen season.

A possible explanation for the different findings described in this and other studies is that the maintenance of antigen-specific Treg cells may depend on continuous antigen exposure. A previous study from our laboratory that examined the capacity of Treg cells to suppress T cell responses to cow's milk showed that Treg cells in children who had outgrown their cow's milk allergy suppressed β -lactoglobulin-mediated T cell proliferation much more efficiently compared with cow's milk-allergic children [16]. However, this difference was only observed when children were reintroduced to milk in their diet for 1 week, which might suggest that antigen-specific Treg cells were generated or became functional during 1 week of provocation. This phenomenon is also supported by recent work from the Akbar group [17]. They show that most Treg cells specific for foreign antigens develop in the periphery in response to antigen exposure and are short-lived unless the antigen persists. These results may suggest that allergen-specific Treg cells induced during the pollen season disappear rapidly after the pollen season, most likely due to apoptosis. This hypothesis also agrees well with results from an asthma model in experimental rats showing that the accumulation of Foxp3⁺ Treg cells depends on continuous local allergen challenge [18]. The suggestion that human Treg cells comprise a dynamic population, which rapidly disappear when antigen is removed, may explain why we did not detect any Treg activity in our out-of-season experiments.

T cell populations with regulatory capacity are heterogeneous, acting through both cell-cell-mediated and

soluble factors. It has been shown that IL-10-producing antigen-specific Treg cells are increased in non-atopics, and that the balance between the number of these cells and Th2 cells is decisive for the development of allergy [19]. Whether this population is within the CD25⁺ subset is controversial [20], and the possibility exists that these cells were not depleted in our experiments. However, we only detected very low levels of IL-10 in the allergen-exposed cultures, which is in agreement with other similar studies [9, 11].

The above-mentioned data clearly demonstrate that several factors must be carefully considered when designing experiments to elucidate the role of Treg cells in allergy, such as selection of patients, type of allergen, allergen dose, environmental exposure during the experimental period and precise definition of Treg cells.

Regarding the latter, we took advantage of novel information showing that CD25⁺ regulatory and effector T cells could be separated based on their expression level of CD127, activated T cells being CD127^{hi}, whereas Treg cells are CD127^{lo} [12, 13]. There was no difference in the numbers of these subsets between allergic patients and non-atopic controls, and the removal of these cells did not increase the proliferation or cytokine production in either of the groups. It will be of great interest to elucidate the role of these different T cell subsets during the pollen season.

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