Trained immunity and tolerance in innate lymphoid cells, monocytes, and dendritic cells during allergen-specific immunotherapy

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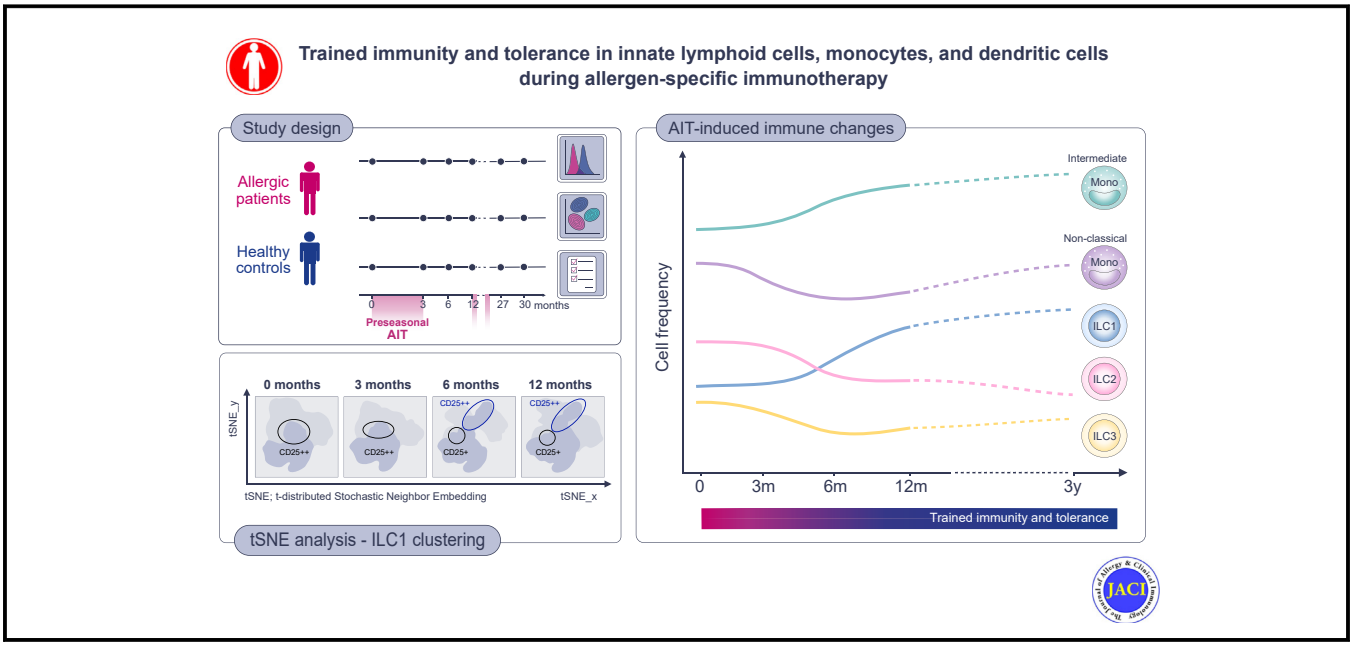
GRAPHICAL ABSTRACT



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Background: Despite the efficacy of allergen-specific immunotherapy (AIT), the role of trained immunity and tolerance in this process has not been elucidated.
Objective: Here, we have performed a comprehensive longitudinal analysis of the systemic innate immune cell repertoire during the course of AIT.
Methods: Patients with allergy received standard preseasonal subcutaneous AIT with allergoids to birch and/or grass. Healthy controls were monitored without any intervention. Flow cytometry of innate lymphoid cell (ILC), natural killer cell, monocyte cell, and dendritic cell (DC) subsets was performed at baseline, 3 months (birch season), 6 months (grass seasons), and 12 months after the therapy in patients or at similar seasonal time points in controls. Additional analyses were performed in the third-year birch and grass season.
Results: We observed a durable decrease in group 2 ILCs and an increase of group 1 ILCs after AIT, with dynamic changes in their composition. We found that an expansion of $CD127^{+}CD25^{++}$ clusters caused observed shifts in the heterogeneity of group 1 ILCs. In addition, we observed development of $CD127^{+}CD25^{++}$c-Kit^{+} group 3 ILC clusters. Moreover, we found an increase in the number of intermediate monocytes in parallel with a reduction in nonclassical monocytes during the first year after AIT. Classical and intermediate monocytes presented significant heterogeneity in patients with allergy, but AIT reduced the HLA-DR^{++} clusters. Finally, an increase in plasmacytoid DCs and $CD141^{+}$ myeloid DCs was observed in individuals with allergy, whereas the number of $CD1c^{+}$ myeloid DCs was reduced during the first year of AIT.
Conclusion: AIT induces changes in the composition and heterogeneity of circulating innate immune cells and brings them to the level observed in healthy individuals. Monitoring of ILCs, monocytes, and DCs during AIT might serve as a novel biomarker strategy. (J Allergy Clin Immunol 2021;147:1865-77.)

Key words: *Allergen immunotherapy, innate immune cells, antigen-presenting cells, monocytes, DCs, ILC, NK cells*

Abbreviations used
AIT: Allergen-specific immunotherapy
CSMS: Combined symptoms and medication score
DC: Dendritic cell
ILC: Innate lymphoid cell
ILC1: Group 1 innate lymphoid cell
ILC2: Group 2 innate lymphoid cell
ILC3: Group 3 innate lymphoid cell
lin: Lineage
mDC: Myeloid dendritic cell
NK: Natural killer
pDC: Plasmacytoid dendritic cell
tSNE: T-distributed stochastic neighbor embedding

Currently, allergen-specific immunotherapy (AIT) remains the only available causative treatment for patients with allergic diseases such as allergic rhinitis and allergic asthma. AIT is based on long-term administration of allergen preparations; it mediates suppression of humoral and cellular effectors. In fact, the vast majority of available studies focus on the mechanisms of adaptive immune responses, mostly T- and B-cell responses, whereas innate immune mechanisms remain elusive.[1] Innate immune cells such as innate lymphoid cells (ILCs), cytotoxic ILCs (natural killer [NK] cells), monocytes/macrophages, and dendritic cells (DCs) orchestrate adaptive immune responses through indirect (soluble factor release) and direct (cell-to-cell interaction) mechanisms.[2,3] Moreover, functional reprogramming of innate immune cells caused by their initial activation may lead to a more intense (trained immunity) or less intense (trained tolerance) response toward the second challenge and is referred to as innate immune memory.[4-6] It is possible that innate memory may represent one of the mechanisms of successful AIT and should be associated with systemic changes in the composition and phenotype of different cell subsets. Therefore, understanding the role of various innate immune cells during the course of AIT will help to fully elucidate complex mechanisms providing long-lasting tolerance to allergens.

On the basis of specific patterns of cytokine production, membrane markers, and transcription factor expression, ILCs are classified into 3 functionally distinct subsets, namely, group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s) , which resemble T_H1, T_H2, and T_H17 subsets, respectively.[7] In addition, thanks to the current advances in single-cell sequencing, even greater heterogeneity of tissue and peripheral blood ILCs has been reported.[8-10] ILCs play a crucial function in the early stage of antimicrobial response, tissue repair, and preservation of epithelial integrity.[11-14] ILC2s have essential functions in allergic diseases. Increased numbers of these cells are observed in peripheral blood after allergen challenge, and they are regulated during AIT.[15-19] Thus, the role of ILCs in allergic inflammation, as well as their phenotypical and functional heterogeneity, need further evaluation.

Similar to ILCs, NK cells possess some functional similarities with T cells and may regulate functions of other immune cells. Two distinct subsets of circulating NK cells can be distinguished on the basis of their expression of surface markers, namely, $CD56^{+}CD16^{++}$ and $CD56^{++}CD16^{+/low}$ NK cells. Notably, $CD56^{+}$ NK cells represent the predominant subset (≤90%) on the periphery; they act mainly as cytotoxic cells and produce low levels of cytokines, whereas $CD56^{++}$ NK cells are recognized as cytokine producers.[20] Interestingly, NK cells were reported to be activated by allergen extracts in a nonspecific manner, and they can promote a type 2 immune response and eosinophilic inflammation.[21]

Monocytes and DCs are antigen-presenting cells, and they link innate and adaptive immune responses. Notably, both subsets may act as activators or regulators of immune responses depending on their antigen-presenting capacities, associated with the expression of checkpoint molecules and cytokine profiles.[22-24] In fact, they have been proposed as crucial mediators of immunologic tolerance,[25-27] also in response to allergens in allergic rhinitis[28] and in fatal asthma in children.[29]

Monocytes represent a heterogeneous cell population in terms of phenotype and biologic properties. According to the differences in CD14 (LPS receptor) and CD16 (Fcγ receptor III) expression, 3 functionally distinct subsets exist in humans, namely, classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and nonclassical ($CD14^{+}CD16^{++}$)

monocytes. Nonclassical monocytes are considered proinflammatory cells that subsequent to stimulation, release high amounts of proinflammatory cytokines, including TNF, and they possess high cytotoxic and phagocytic activities. Elevated frequencies of these cells are observed in proinflammatory diseases, infections, and sepsis.[30] In contrast, intermediate monocytes have been referred to as anti-inflammatory because following stimulation, they constitute the principal source of IL-10 among all 3 subsets[31,32]; they also play essential roles in vascularization, tissue repair, and regeneration processes, as well as in induction of tolerance.[26,33,34] Notably, it is well established that monocytes may serve as macrophage and DC progenitors. In fact, both $CD16^+$ subsets are referred to as activated monocytes and are considered a more mature form of monocytes when compared with the classical subset, which can subsequently differentiate into monocyte-derived DCs.[31,35]

It is generally accepted that 3 distinct DC subsets can be distinguished in peripheral blood in humans, namely, plasmacytoid DCs (pDCs), ($CD303^+$ pDCs), $CD1c^+$ myeloid DCs (mDCs), and $CD141^+$ mDCs, with an additional extent of heterogeneity being discovered as a result of single-cell sequencing.[32,36] pDCs are considered an immature form of DCs because after a contact with antigen, which acts as a maturation signal, they can present antigen to the T cells immediately in the lymph nodes. The remaining 2 subsets of mDCs show features of both immature DCs and $CD303^+$ DC precursors.[32] Interestingly, it has been previously postulated that mDCs are involved mainly in the development of T_H2 responses, whereas pDCs may induce antiviral responses or orchestrate tolerance development by induction of regulatory T cells.[37-39]

In this study, we analyzed dynamic changes in the systemic innate immune responses at several time points during the course of preseasonal subcutaneous allergen-specific immune therapy for birch and grass pollens and compared them with the physiologic responses in healthy controls.

METHODS

Patients and healthy controls

Subjects allergic to grass and/or birch pollen and healthy allergy-free nonatopic controls were recruited in spring and summer 2015 at Inselspital Bern (Bern, Switzerland). The study was initiated from December 2015 to January 2016, when patients received preseasonal subcutaneous immunotherapy to birch and/or grass. All subjects lived in the Bern region. The patients were clinically diagnosed with pollen allergic rhinitis (symptoms in birch and/or grass season on the basis of positive skin prick test results and the presence of allergen-specific IgE, as assessed with Immunocap (Phadia AB, Uppsala, Sweden). Some patients had concomitant well-controlled allergic asthma.[40] The patients did not have any other clinically significant concomitant acute or chronic diseases. Further exclusion criteria were pregnancy, noncompliance, inability to follow the study procedures, drug or alcohol abuse, or participation in a study involving investigational drug use 1 month before and during the course of this study. A total of 12 nonatopic, allergy-free healthy control subjects from the same region as the patients with allergy were followed at the same seasonal time points as the patient population to control for potential seasonal changes in the innate immune cell populations. Detailed demographic and clinical characteristics of the study participants are shown in Table I. This study has been reviewed and approved by the ethics committee of the Canton of Bern (Switzerland). Each participant was familiarized with the objectives of the study and provided written consent.

Preseasonal birch and/or grass pollen immunotherapy, study design, and sampling

All of the patients with allergy started the first course of subcutaneous preseasonal AIT at some point between the end of November 2015 and the beginning of January 2016, and again in the same format in the following 2 years (see Fig E1, *A* in this article's Online Repository at www.jacionline.org). According to their diagnosis, patients received subcutaneous injections of depot allergen extracts of grass and/or tree pollen preparations containing the adjuvant alum (Allergopharma Merck, Reinbek, Germany) (see Fig E1, *B* and *C*). The standard therapy protocol consisted of increasing concentrations in a total of 7 to 9 injections (see Fig E1, *B* and *C*) given within first 2 to 3 months. For patients receiving both tree and grass pollen AIT, the preparations were injected separately, each into opposite arms. Birch and grass pollen counts were retrieved from the Federal Office of Meteorology and Climatology MeteoSwiss (Zurich-Airport, Switzerland) (see Fig E1, *D* and *E*). Blood samples were collected at 4 time points—time point 0 (immediately before the start of preseasonal AIT), after 3 months (at the end of preseasonal therapy [at the peak of birch pollen season]), after 6 months (at the peak of grass pollen season), and at 12 months (out of season) for patients—and at similar time points for the healthy controls (see Fig E1).[41] In the follow-up, we also collected blood samples from a small subset of patients at 27 and 30 months after the start of AIT.

Venous blood samples were collected at Inselspital Bern and transported to the Swiss Institute for Allergy and Asthma Research (Davos, Switzerland) at ambient air temperature within 6 hours after venipuncture. The blood was further processed for direct whole blood sample staining with flow cytometry antibodies, plasma collection, PBMC isolation, and subsequent flow cytometry with different panels.

Clinical response to AIT was assessed according to the European Academy of Allergy and Clinical Immunology (EAACI) guidelines.[42] Combined symptom and medication scores (CSMSs) were recorded by the CSMS questionnaires in the doctor's office in the season before, and at each study visit, together with sampling. Nonresponders to AIT were defined as those subjects who had 30% or less improvement in their CSMS as compared with during the season preceding initiation of AIT. Total levels of IgE, IgE specific to *Phleum pratense* 1 combined with *Phleum pratense* 5b, and IgE specific to *Betula verrucosa* 1 antibodies were measured in plasma by using Immunocap.

Flow cytometry

For ILCs analysis, 20 million freshly isolated PBMCs were stained with a panel of anti-human antibodies. All antibodies with their clones and manufacturers are presented in Table E1 (in this article's Online Repository at www.jacionline.org). ILCs were characterized as viable, lineage-negative (lin^-) ($CD3^-CD4^-CD19^-CD14^-CD16^-CD1a^-CD11c^-CD34^-CD94^-CD123^-CD303^-Fc\varepsilon RIa^-$) $CD127^+$ cells and further discriminated as $CRTH2^+$ILC2, $CRTH2^-$c-Kit^- ILC1, or $CRTH2^-$c-Kit^+NKp44^- ILC3 (applied gating strategy is presented in Fig E2 (in this article's Online Repository at www.jacionline.org).

For immunophenotyping of monocytes, DCs, and NK cells, 200 μL of fresh whole blood was stained with a panel of anti-human antibodies according to the stain-lyse-wash protocol. Briefly, antibodies were added to 200 μL of whole blood samples, mixed, and incubated for 30 minutes at room temperature in the dark. Next, 2 mL of lysing buffer (Becton Dickinson) was added for red blood cell lysis. Finally, the samples were washed in phosphate-buffered saline (PBS) and analyzed by using flow cytometry. Monocytes were classified on the basis of morphology and fluorescent markers (viable $CD3^-CD19^-CD14^+$). Subpopulations were identified as classical, intermediate, and nonclassical monocytes by their expression of $CD14^{++}CD16^-$, $CD14^{++}CD16^+$, or $CD14^+CD16^{++}$, respectively (see the applied gating strategy in Fig E3 in this article's Online Repository at www.jacionline.org). DC subsets were classified as viable $CD3^-CD19^-HLA\text{-}DR^+$ cells and on the basis of expression of specific markers, namely, $CD123^{++}CD303^+$ for pDCs, $HLA\text{-}DR^{++}CD1c^+$ for $CD1c^+$ mDCs, and $CD14^-CD141^{++}$ for $CD141^+$ mDCs (see the applied gating strategy in Fig E4 in this article's Online Repository at www.jacionline.org). NK cells were classified as viable $CD19^-CD4^-CD14^-CD56^{+/++}CD16^{+/++}$ cells (see

TABLE I. Demographic and clinical characteristics of the study participants

Characteristics	Patients with allergy (n = 13)	Healthy controls (n = 12)	*P* value
Demographic characteristics			
Age (y), median (range)	27 (19-36)	35.5 (23-63)	.0222
Sex, female/male (no./no.)	5/7	10/2	.0894
Weight (kg), median (range)	77 (63-100)	66.5 (53-102)	.0612
Height (cm), median (range)	172 (162-187)	169 (153-188)	.2295
Clinical data			
Allergic rhinitis (yes/no)	13/0	0/12	<.0001
Asthma (yes/no)	8/5	0/12	.0016
Positive SPT result (yes/no)	13/0	0/12	<.0001
Total IgE level (kU/L), median (range)	106 (28-274)	26 (10-73)	.0003
Phl p 1 + Phl p 5 IgE level (kU/L), median (range)	9.88 (1.59-68.9)	nd	<.0001
Bet v 1 IgE level (kU/L), median (range)	2.99 (0.1-36.4)	nd	.0005
FEV_1 (% predicted), median (range)	101 (79-122)	n/a	n/a
Allergen immunotherapy			
Birch (yes/no)	10/3	n/a	n/a
Grass (yes/no)	12/1	n/a	n/a
Clinical nonresponders to birch AIT, no. (%)*	2 (15.4)	n/a	n/a
Clinical nonresponders to grass AIT, no. (%)*	3 (23.1)	n/a	n/a

Bet v, *Betula verrucosa*; *n/a*, not applicable; *nd*, not determined; *Phl p*, *Phleum pratense*; *SPT*, skin prick test.
Data were analyzed by using the Mann-Whitney and Fisher exact test, as appropriate.
*Nonresponders to AIT were assessed as having a 30% or lower decrease in CSMS, as compared with the level during the appropriate pollen season preceding initiation of AIT.

the applied gating strategy in Fig E5 in this article's Online Repository at www.jacionline.org).

All specimens were acquired by using a FACS ARIA III flow cytometer followed by data analysis in FlowJo 10.1 software (TreeStar, Ashland, Ore). Classical gating and t-distributed stochastic neighbor embedding (tSNE) algorithms were used to assess the subpopulations. The frequency of each analyzed subpopulation was calculated in the parent population in gating, unless otherwise specified. Total cell number was recalculated per microliter of whole blood, on the basis of the initial values measured by using a Sysmex cell counter.

For 2-dimensional reduction of manual gates FlowJo tSNE plugin software was used. The data from each time point of each individual patient were evenly downsampled to ensure that tSNE analysis would be performed at the same number of events, namely, the highest event number in the particular gate available in all time points. Next, all files were concatenated into a new data file and the tSNE algorithm was applied. The following settings were used for the tSNE algorithm: perplexity, 20 (P_20); learning rate, 200 (E_200); iterations, 600 (I_600); and theta, 0.5 (T_0.5).

Statistics

Statistical analysis was conducted by using GraphPad Prism 7 software (GraphPad Software, La Jolla, Calif). Categoric variables were analyzed with the Fisher exact test, whereas continuous variables were analyzed with the Mann-Whitney *U* test, as appropriate. The Wilcoxon test was used to compare changes between different time points. The differences were considered statistically significant at a *P* value less than .05.

RESULTS

ILC2s and ILC3s decrease during the course of AIT

First, we investigated the effect of AIT on the frequency and absolute numbers of the 3 main and best-characterized subsets of ILCs, namely ILC1, ILC2, and ILC3 (Fig 1, *A*). Three types of comparisons were made: (1) first-year time points were compared with baseline, (2) third-year time points after initiation of AIT were compared with the first-year time points; and (3) patients with allergy were compared with the healthy controls at the same time points. We found increased frequencies and numbers of ILC1s at the 6- and 12-month time points (Fig 1, *B* and see Fig E6, *A* in this article's Online Repository at www.jacionline.org). In contrast, the frequency of ILC2s was decreased after 12 months. Interestingly, the frequency of ILC2s decreased most between the 6- and 12-month time points; however, only a trend can be observed in ILC2 numbers (Fig 1, C and see Fig E6, *A* in this article's Online Repository at www.jacionline.org). ILC3 frequency also decreased after 12 months (Fig 1, *D*).

Importantly, we noticed that the observed first-year effects of AIT on ILC subsets remained stable or were even more profound in the third year (see Fig E6, *B*). In particular, we observed a further increase in the frequency of ILC1s and a further decrease in the frequency of ILC2s. We found no changes in the frequency of ILC3s between the first and third year of AIT in the birch pollen season, whereas the frequency of ILC3s in the grass pollen season was increased (at the 6- vs 30-month time points).

Having found the time course changes in ILC subsets during the first year after AIT, we evaluated the differences between the frequency of the analyzed cells in individuals with allergy during the course of AIT and that in the matched controls at the same seasonal time points. The healthy controls showed no seasonal changes in any of the analyzed ILC subsets (see Fig E6, *C*). In a comparison of controls with patients at the same time points, a lower frequency of ILC1s in patients with allergy at baseline and 6 months after initial treatment was observed (Fig 1, *E*). After 12 months of treatment, there was no difference in the ILC1 frequency between patients and controls. An elevated frequency of ILC2s in the donors with allergy was demonstrated at baseline and at 6 months (in the grass pollen season); however, they became equally frequent in patients and controls after 12 months (Fig 1, *F*). There were no differences in the frequency of ILC3s between patients and controls at any of the analyzed seasonal time points (Fig 1, *G*).

Next, we used the tSNE algorithm to analyze heterogeneity of ILC subsets clustering at baseline and their changes over time

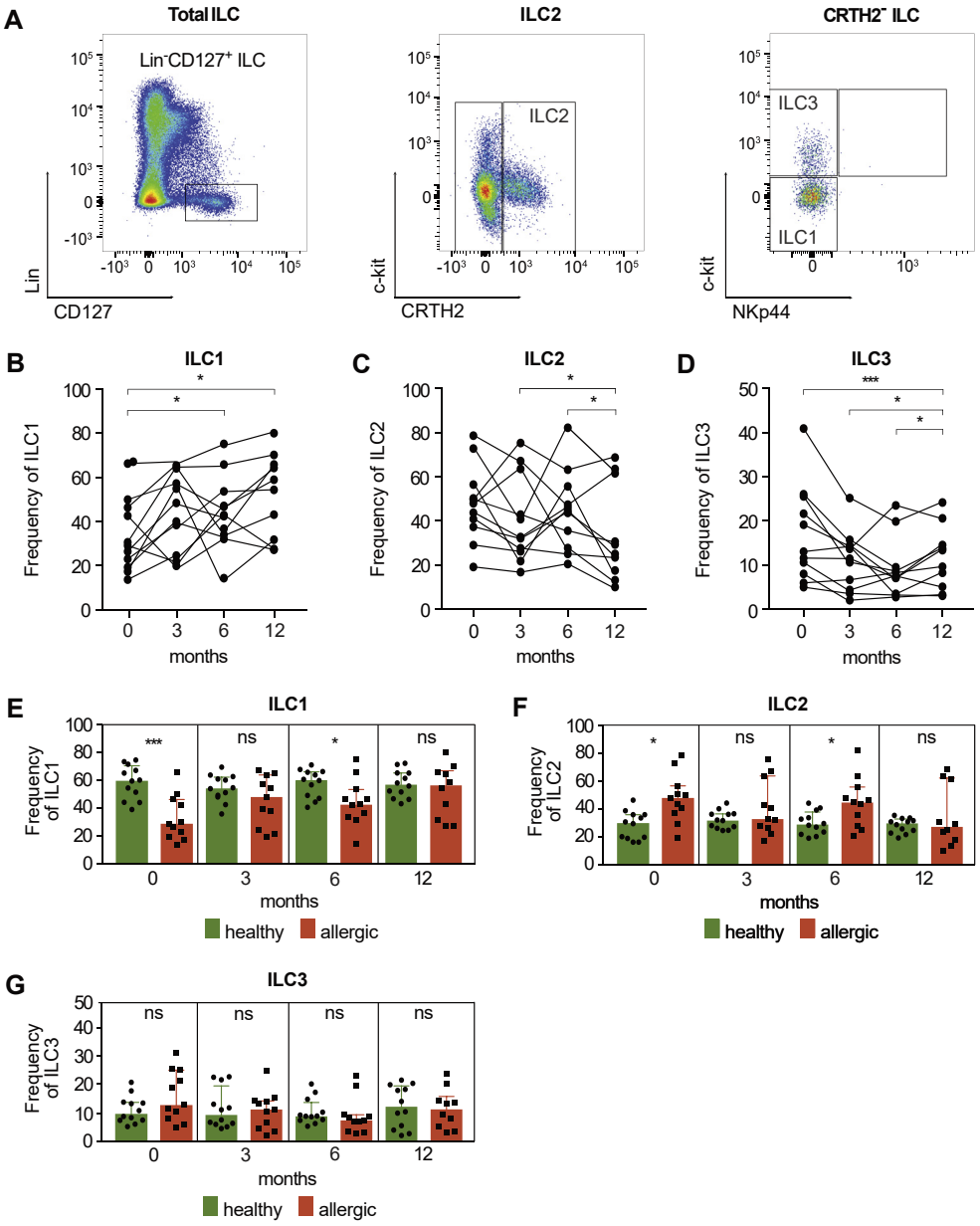

FIG 1. AIT-induced decrease in ILC2s and ILC3s. **A,** Representative gating of ILC1s, ILC2s, and ILC3s among lin⁻CD127⁺ ILCs. Summary of flow cytometry analyses of AIT-induced changes in the frequency of ILC1s (**B**), ILC2s (**C**), and ILC3s (**D**) in patients with allergy during the course of AIT (n = 11 [the Wilcoxon test was used]). Each ILC subset is shown as a percentage in the lin⁻CD127⁺ gate. Comparison of seasonal changes of ILC1s (**E**), ILC2s (**F**), and ILC3s (**G**) between allergy-free healthy controls (n = 12), and patients with allergy (n = 11) during the course of AIT. The Wilcoxon test was used to compare differences between time points. The Mann-Whitney *U* test was used to compare differences between the controls and patients who received AIT. **P* < .05; ***P* < .01; ****P* < .001.

(Fig 2). As expected, the analyzed ILC subpopulations clustered separately, confirming use of the correct gating strategy (see tSNE ILC subsets in Fig E2). As a result of in-depth analysis of cluster heterogeneity with subsequent quantification of the newly identified subsets by classical gating, we noticed intriguing changes in the ILC1 and ILC3 phenotypes, but not in the ILC2 phenotypes over time. Briefly, AIT induced an increase in the numbers of ILC1 clusters with higher CD25 expression (Fig 2, *A*), thus making their signature comparable to that of allergy-free individuals at 12 months (see Fig E7 in this article's Online Repository at www.jacionline.org). In fact, there was a significant elevation of the frequency of the $CD127^{+}CD25^{++}$ cluster at the 6-month time point (Fig 2, *B*). In contrast, the frequency of the$CD127^{++}CD25^{++}$ subset decreased at 12 months when compared with baseline (Fig 2, *C*). Interestingly, we found that ILC2s were quite heterogenous, but there were no significant changes in the presence (Fig 2, *D* and see Fig E8 in this article's Online Repository at www.jacionline.org) or frequency of ILC2 clusters (Fig 2, *E* and *F*) over time. The signature of the ILC3 subpopulations in individuals with allergy was less complex at

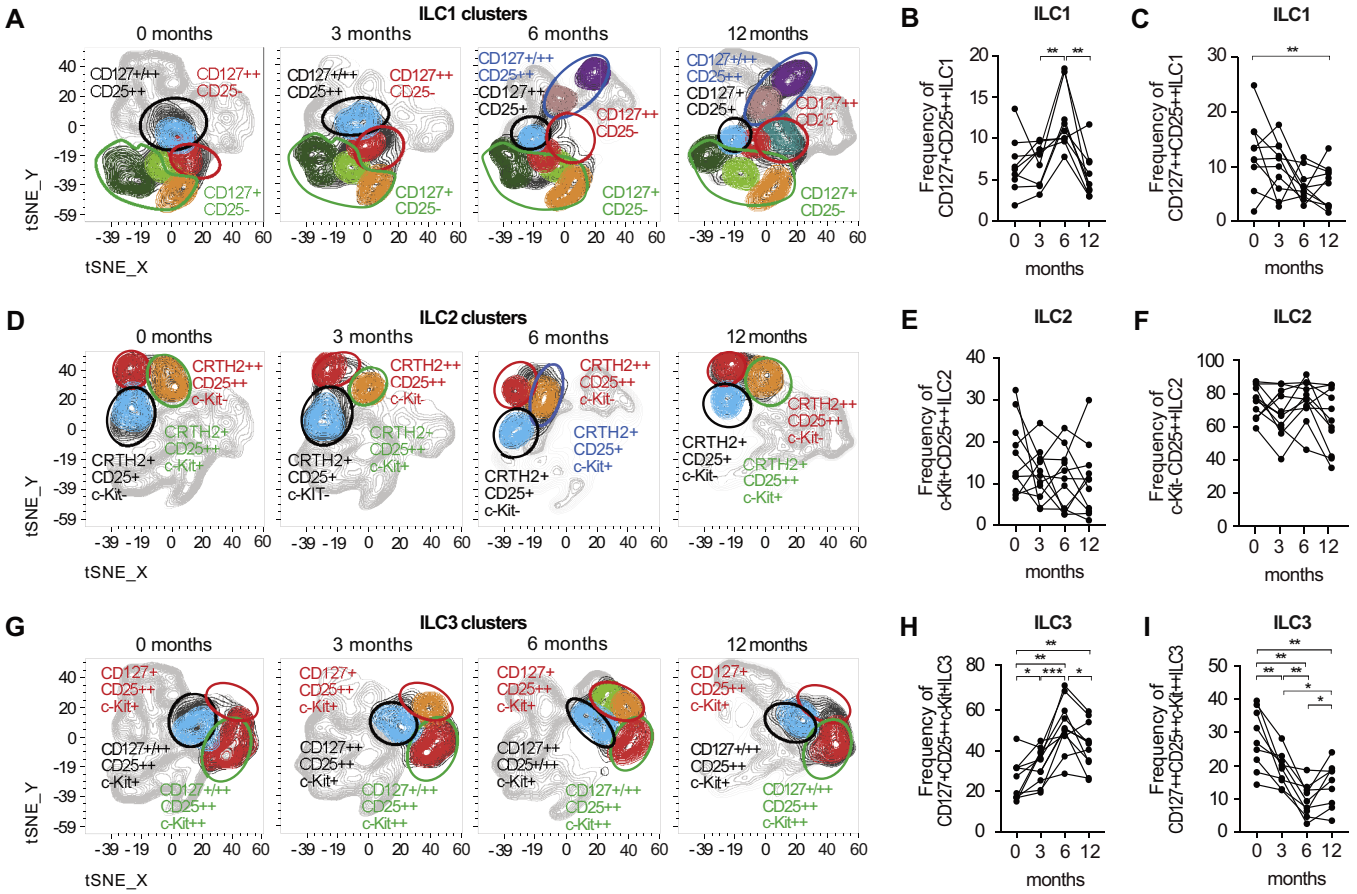

FIG 2. AIT-induced changes in the heterogeneity of circulating ILCs. Representative tSNE 2-dimensional plots showing changes in the complexity and clustering of different circulating ILC subsets at each analyzed time point in patients with allergy who received AIT. **A,** ILC1 clustering and quantification of $CD127^{+}CD25^{++}$ ILC1 clusters (**B**) and $CD127^{++}CD25^{++}$ ILC1 clusters (**C**) (n = 10). **D,** ILC2 clustering and quantification of $c\text{-}Kit^{+}CD25^{++}$ ILC2 clusters (**E**) and $c\text{-}Kit^{-}CD25^{++}$ ILC2 clusters (**F**) (n = 11). **G,** ILC3 clustering and quantification of $CD127^{+}CD25^{++}c\text{-}Kit^{+}$ (**H**) and $CD127^{++}CD25^{++}c\text{-}Kit^{++}$ ILC3s (**I**) (n = 11). For tSNE analysis, the cells were gated as described in Fig E2 and a 2-dimensional reduction was performed from the $lin^{-}CD127^{+}$ gate (total ILCs) for NKp44, CRTH2, CD25, CD127, c-Kit, and ILT3 expression. The following settings were applied for the tSNE algorithm: perplexity, 20 (P_20); learning rate, 200 (E_200); iterations, 1000 (I_1000); and theta, 0.5 (T_0.5). Graph overlays were performed to visualize changes in cluster complexity of the different ILC subsets, namely, ILC1s (**A**), ILC2s (**D**), and ILC3s (**G**). For novel subset quantification, manual gating was applied. The Wilcoxon test was used to compare differences between time points. $*P < .05$; $**P < .01$; $***P < .001$.

baseline (Fig 2, *G*) and differed significantly from that of the controls (see Fig E9 in this article's Online Repository at www.jacionline.org). At 3 months and at 6 months after initiation of AIT, there was an increase in the complexity of the ILC3 clusters, associated with the expansion of $CD127^{+}CD25^{++}c\text{-}Kit^{+}$ (Fig 2, *H*) and a decrease of $CD127^{++}CD25^{++}c\text{-}Kit^{++}$ cells (Fig 2, *I*). A detailed description of ILC clusters can be found in the Results section of the Online Repository (at www.jacionline.org) and also in Figs E7 and E9.

Taken together, our data suggest that AIT limits the numbers of circulating ILC2s and ILC3s in favor of an expansion of the number of ILC1s. In addition, AIT also induces an increase in ILC1 and ILC3 heterogeneity, bringing their phenotypes closer to that of the healthy controls. This may reflect trained tolerance to reduce seasonal induced allergic inflammation.

AIT does not affect the composition of circulating NK cells

Having found AIT-induced changes in the composition of ILCs, we next analyzed changes in the frequency and numbers of ILCs with cytotoxic potential, namely, $CD56^{+}CD16^{++}$ (CD56 dim) and $CD56^{++}CD16^{+/-}$ (CD56 bright) NK cells (Fig 3, *A*). We observed no changes in the frequency of NK cells in patients with allergy who were undergoing AIT, mainly owing to huge individual variations (Fig 3, *B* and *C*). Similarly, we found no significant changes in the numbers of both analyzed subsets (see Fig E10, *A* in this article's Online Repository at www.jacionline.org). As an interesting finding that requires further investigation, allergy-free individuals showed a significant decrease in the frequencies of both CD56 bright and CD56 dim NK cells during the birch season (3 months [see Fig E10 in this article's Online Repository at www.jacionline.org).

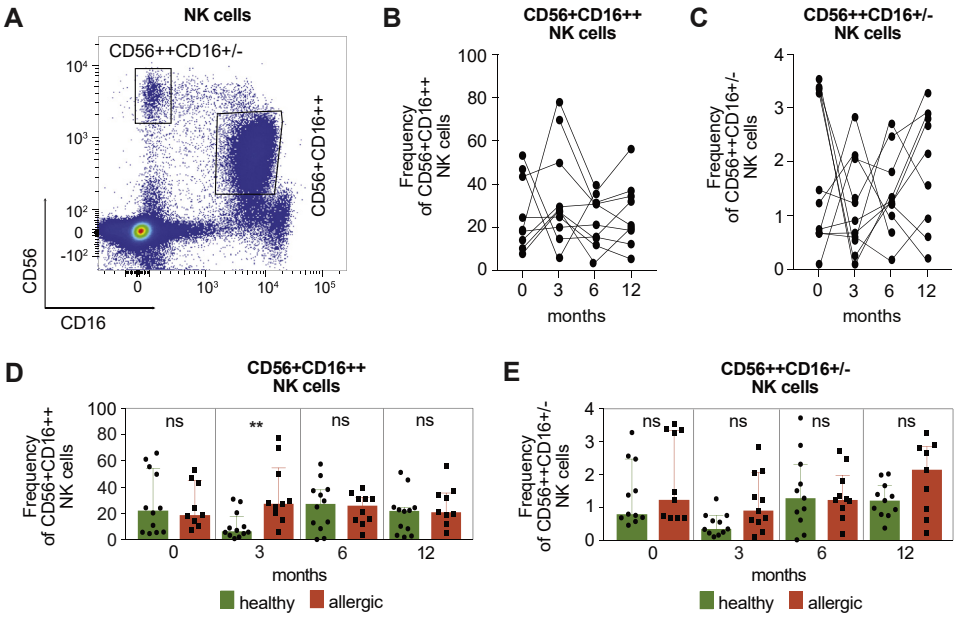

FIG 3. AIT does not affect the composition of circulating NK cells. **A,** Representative gating for $CD56^{++}CD16^{+/-}$ and $CD56^{+}CD16^{++}$ NK cells. Summary of flow cytometry analyses of AIT-induced changes in the frequency of $CD56^{+}CD16^{++}$ NK (**B**) and $CD56^{++}CD16^{+/-}$ (**C**) cells in patients with allergy during the course of AIT (n = 11). Comparison of seasonal changes of $CD56^{++}CD16^{++}$ (**D**) and $CD56^{+}CD16^{+/-}$ (**E**) NK cells between allergy-free healthy controls (n = 12), and patients with allergy during the course of AIT (n = 11). The Wilcoxon test was used to compare differences between time points, and the Mann-Whitney *U* test was used to compare differences between controls and patients who received AIT. $^{**}P < .01$.

Consequently, we found a higher frequency of $CD56^{+}CD16^{++}$ NK cells in patients with allergy at the 3-month time point, as compared with healthy controls, whereas no differences were observed at 6 and 12 months (Fig 3, *D*). Moreover, no differences in the frequency of $CD56^{++}CD16^{+/-}$ NK cells were observed between patients receiving AIT and healthy controls at the same seasonal time points (Fig 3, *E*).

In summary, AIT did not change the systemic distribution of cytotoxic ILCs. It seems, therefore, that circulating NK cells may play a minor role in the AIT-reinduced tolerance to allergens.

Monocytes shift from proinflammatory toward anti-inflammatory phenotypes during the course of AIT

Next, we aimed to assess the composition of circulating innate immune cells with antigen-presenting capacities. First, we analyzed the composition and heterogeneity of peripheral blood monocytes (Fig 4, *A*). We observed a substantial decrease in the frequency of nonclassical $CD14^{+}CD16^{++}$ monocytes already at 3 months (Fig 4, *B*). This decrease was even more profound at 6 months (Fig 4, *B* and see Fig E11, *A* in this article's Online Repository at www.jacionline.org). Moreover, there was a steady increase in the frequency of intermediate $CD14^{++}CD16^{+}$ monocytes during the course of AIT, reaching a maximum at 12 months (Fig 4, *C* and see Fig E11, *A*), whereas the frequency of classical $CD14^{++}CD16^{-}$ monocytes showed alterations reflecting changes in the other subpopulations (Fig 4, *D* and see Fig E11, *A* in this article's Online Repository at www.jacionline.org).

Notably, in contrast to the effects of AIT on the ILC subsets, the effects of AIT on the composition of different monocyte subsets was less stable over time (see Fig E11, *B* in this article's Online Repository at www.jacionline.org). In contrast to the early (first-year) effects of AIT, we found a significant increase in the frequency of $CD14^{+}CD16^{++}$ nonclassical monocytes in the third-year pollen season. Simultaneously, the frequency of $CD14^{++}CD16^{+}$ intermediate monocytes did not increase at that time (see Fig E11, *B*).

Next, we compared the frequency of different monocyte subsets between patients with allergy and healthy controls at the same time points. Interestingly, we found that there was a slightly reduced frequency of nonclassical monocytes at 12 months in patients (Fig 4, *E*). Surprisingly, no differences were observed in intermediate monocytes (Fig 4, *F*). However, we noticed slight, but significant differences in classical monocytes at 3 and 6 months (Fig 4, *G* and see Fig E11, *C*).

tSNE analysis of the different monocyte subsets demonstrated that all of the analyzed clusters separated from other cell types, confirming their distinct phenotype (see the tSNE APC subsets in Fig E3). We found no change in the complexity of nonclassical monocytes in the patients receiving AIT (Fig 5, *A*) and healthy controls (see Fig E12 in this article's Online Repository at www.jacionline.org), yet there was an increase in the expression of HLA-DR (Fig 5, *B*), with stable expression of CD141 in this subset (Fig 5, *C*). In contrast, we noticed significant heterogeneity in the remaining 2 subsets, namely, intermediate and classical monocytes. Briefly, we observed a reduction of $HLA\text{-}DR^{++}$ clusters in the intermediate monocytes (Fig 5, *D* and see Fig E13 in this article's Online Repository at www.jacionline.org), and we further confirmed it by quantification of HLA-DR (Fig 5, *E*) and CD141 expression (Fig 5, *F*). In classical monocytes, we

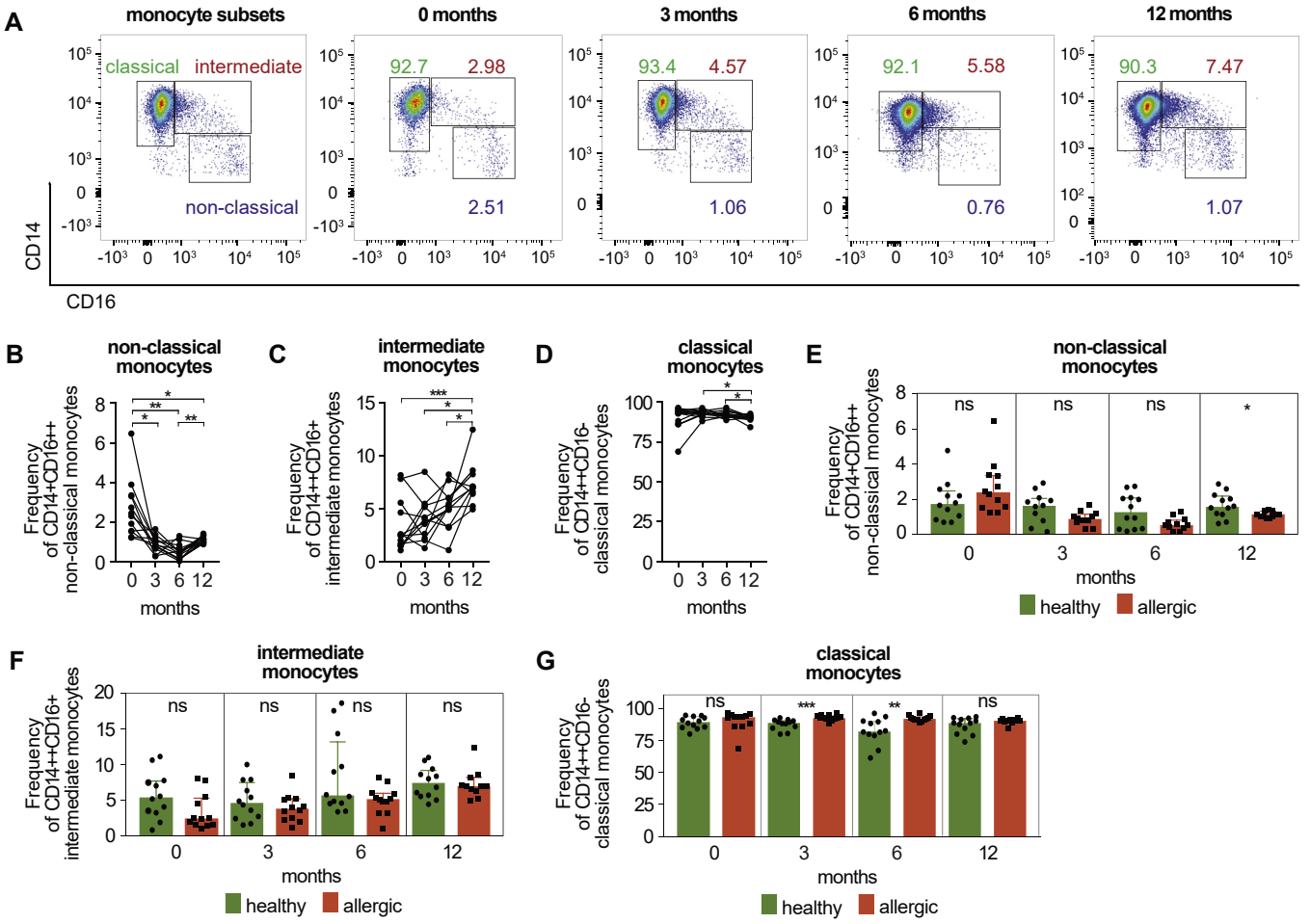

FIG 4. Monocyte shift from proinflammatory toward anti-inflammatory phenotypes during the course of AIT. **A,** Representative gating for $CD14^{++}CD16^{-}$ classical, $CD14^{++}CD16^{+}$ intermediate, and $CD14^{+}CD16^{++}$ nonclassical monocytes and representative dot plots of different monocyte subsets at each analyzed time point. Summary of flow cytometry analyses of AIT-induced changes in the frequency of $CD14^{+}CD16^{++}$ nonclassical (**B**), $CD14^{++}CD16^{+}$ intermediate (**C**), and $CD14^{++}CD16^{-}$ classical monocyte (**D**) subsets in patients with allergy who were receiving AIT (n = 12). Comparison of seasonal changes of $CD14^{+}CD16^{++}$ nonclassical (**E**), $CD14^{++}CD16^{+}$ intermediate (**F**), and $CD14^{++}CD16^{-}$ classical (**G**) monocyte subsets between allergy-free healthy controls (n = 12), and patients with allergy (n = 12) during the course of AIT. The Wilcoxon test was used to compare differences between time points. The Mann-Whitney *U* test was used to compare differences between controls and patients receiving AIT. $*P < .05$; $**P < .01$; $***P < .001$. *ns,* Not significant.

found an expansion of $HLA\text{-}DR^{+}CD141^{+/-}$ clusters at 12 months in patients with allergy (Fig 5, *G* and see Fig E14 in this article's Online Repository at www.jacionline.org). Consequently, there was a slight decrease in HLA-DR expression from 3 months to 12 months (Fig 5, *H*) and a significant decrease of CD141 expression from 6 months to 12 months (Fig 5, *I*). A more detailed description of the different monocyte clusters can be found in the Results section of the Online Repository and in Figs E12 to E14.

In summary, we found that AIT reduced the number of nonclassical monocytes with no changes in their composition. Moreover, we observed an increase in the number of intermediate monocytes in patients during AIT, accompanied by the dynamic changes in the composition of these cells that were associated with the expansion of clusters with lower HLA-DR expression. The observed reduction in HLA-DR expression on intermediate monocytes, together with the increase in the frequency of these cells, complementary with the decrease in nonclassical, proinflammatory monocytes, may reflect a shift toward anti-inflammatory/tolerogenic phenotypes. It indicates a trained, systemic response to limit local inflammation.

$CD1c^{+}$ mDCs, but not $CD141^{+}$ mDCs and pDCs, are downregulated during the course of AIT

Next, we aimed to analyze whether AIT induces changes in the phenotype and composition of peripheral blood DC subsets, namely $CD1c^{+}$ mDCs, $CD141^{+}$ mDCs, and pDCs. In contrast to the different monocyte subsets showing varying expression levels of CD14 and CD16, peripheral blood DCs have been characterized as more distinct subpopulations expressing the characteristic

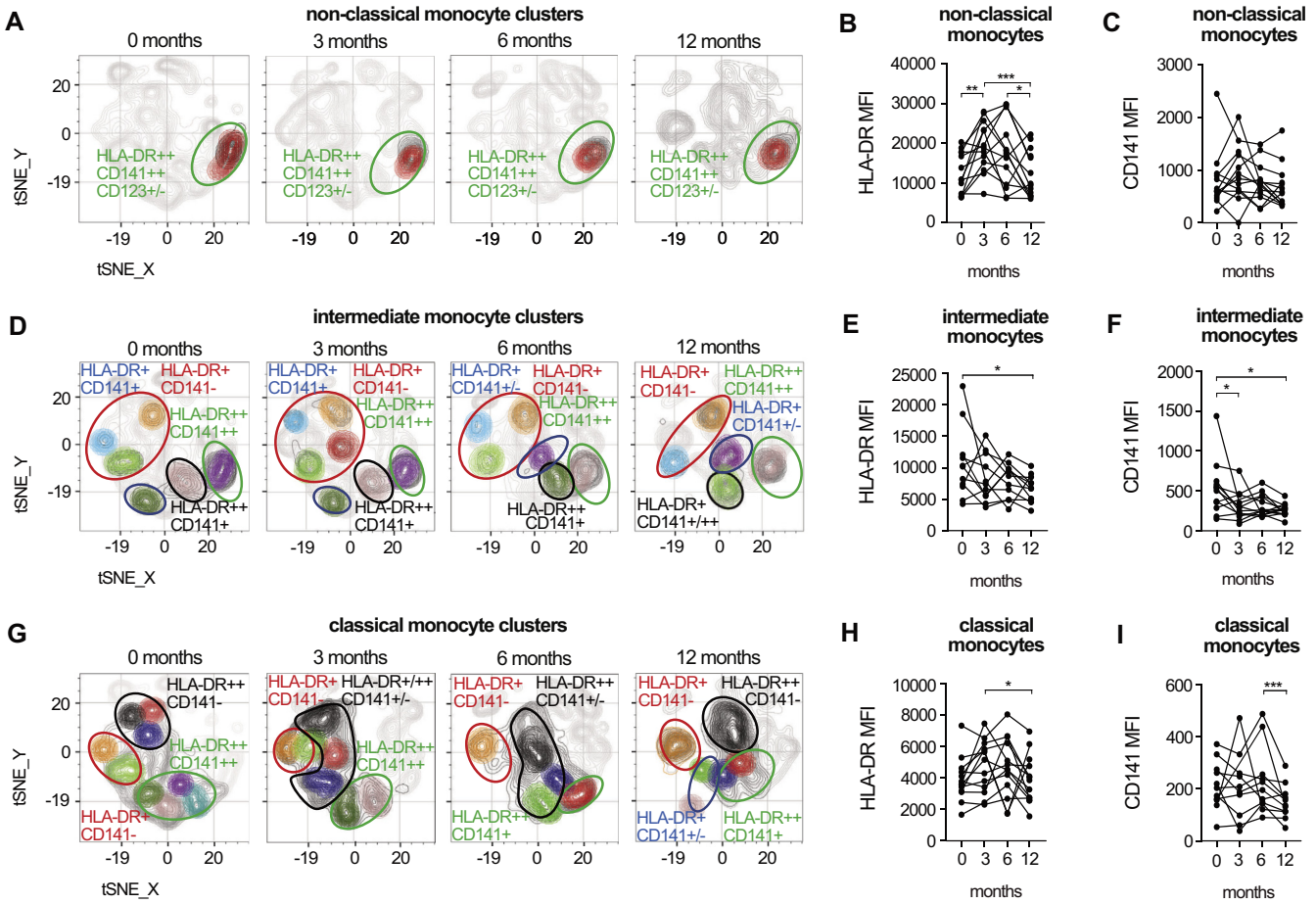

FIG 5. AIT-induced changes in the heterogeneity of different subsets of monocytes. Representative tSNE 2-dimensional plots representing changes in complexity and clustering of different monocytes subsets at each analyzed time point. For tSNE analysis, the cells were gated as follows. First, flow check control was applied to gate out all disruptions in the instrument performance. Next, doublet discrimination was performed according to both forward scatter (FSC-H and FSC-A) and side scatter (SSC-H vs SSC-W), followed by dead, CD3, and CD19 cell discrimination (viability). Next HLA-DR$^+$ cells were gated. Finally, 2-dimensional reduction was performed from HLA-DR$^+$ cells for CD1c, CD14, HLA-DR, CD16, CD123, CD141, and CD303 expression. The following settings were applied for the tSNE algorithm: perplexity, 20 (P_20); learning rate, 200 (E_200); iterations, (I_600); and theta, 0.5 (T_0.5). Graph overlays were performed to visualize changes in cluster complexity: **A,** CD14$^+$CD16^{++} nonclassical monocytes clusters with quantification of HLA-DR (**B**) and CD141 MFI (**C**). **D,** CD14^{++}CD16$^+$ intermediate monocyte clusters with quantification of HLA-DR and CD141 expression (**E**). **G**, CD14^{++}CD16$^-$ classical monocyte clusters and quantification of HLA-DR (**H**) and CD141 **(I)** MFI (n = 11). The Wilcoxon test was used to compare differences between time points. *$P < .05$; **$P < .01$; *** $P < .001$.

pattern of membrane molecules (Fig 6, *A*). Here, we observed an increase in the frequency of pDCs at the 6- and 12-month time points compared with at 3 months (Fig 6, *B*). In addition, we found a substantial decrease in the frequency and numbers of CD1c$^+$ mDCs during the course of AIT when compared with baseline (Fig 6, *C* and see Fig E15, *A* in this article's Online Repository at www.jacionline.org). Similarly to nonclassical monocytes, CD1c$^+$ mDCs decreased significantly after 3 months of AIT and even further after 6 months (Fig 6, *C*). In addition, we observed a slight elevation of the frequency of CD141$^+$ mDCs at 12 months compared with at 3 months (Fig 6, *D*). However, no differences were observed in the numbers of CD141$^+$ mDCs, mainly owing to noticeable individual variations (see Fig E15, *A* in this article's Online Repository at www.jacionline.org).

Similarly to monocytes, the AIT-induced changes in the composition of DCs in the pollen season of the first year seemed to be modified in the subsequent seasons. We found a lower frequency of pDCs in the third-year birch pollen season compared with at 3 months after AIT (see Fig E15, *B*). The frequency of CD1c$^+$ mDCs in the third-year birch and grass pollen seasons remained comparable to the levels observed in the first year after AIT. In contrast, the frequency of CD141$^+$ mDCs decreased significantly in the third-year birch pollen season and was slightly increased in the grass pollen season, but it still remained lower than in the representative seasons of the first year.

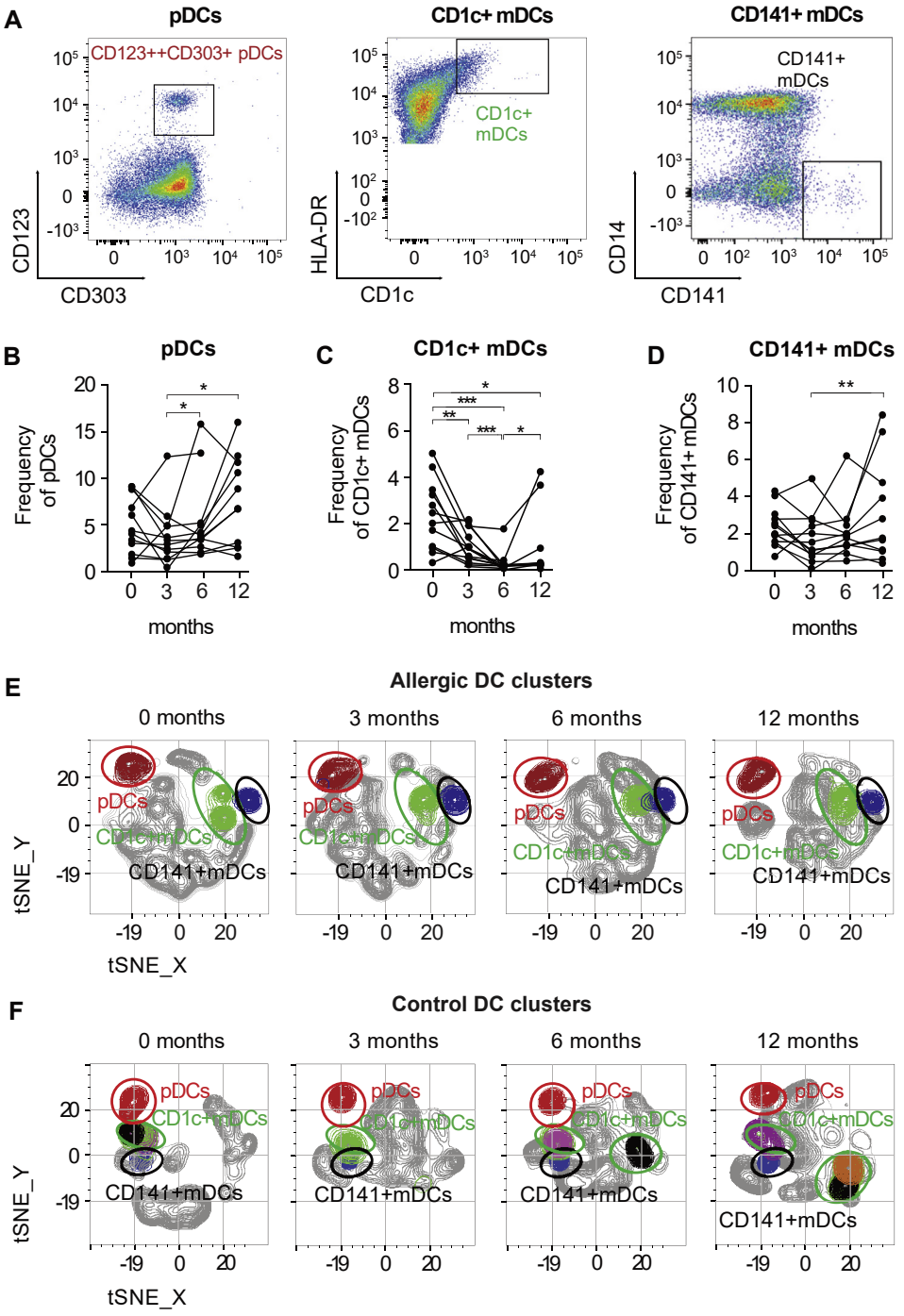

FIG 6. pDCs and CD141^{+} mDCs are upregulated, whereas CD1c^{+} mDCs are downregulated during the course of AIT. **A,** Representative gating for pDCs, CD1c^{+} mDCs, and CD141^{+} mDCs. Summary of flow cytometry analyses of AIT-induced changes in the frequency of CD123^{++}CD303^{+} pDCs **(B)**, HLA-DR^{++}CD1c^{+} mDCs (CD1c^{+} mDCs) **(C)**, and CD14^{-}CD141^{++} (CD141^{+} mDCs) **(D)** in patients with allergy during the course of AIT (n = 12). Representative tSNE 2-dimensional plots representing changes in complexity and clustering of different DC subsets at each analyzed time point in individuals with allergy **(E)** and healthy allergy-free individuals **(F)**. For tSNE analysis, the cells were gated as follows. First, flow check control was applied to gate out all disruptions in the instrument performance. Next, doublet discrimination was performed according to both forward scatter (FSC-H and FSC-A) and side scatter (SSC-H vs SSC-W), followed by dead, CD3, and CD19 cell discrimination (viability). Next, HLA-DR^{+} cells were gated. Finally, 2-dimensional reduction was performed from HLA-DR^{+} cells for CD1c, CD14, HLA-DR, CD16, CD123, CD141, and CD303 expression. The following settings were applied for the tSNE algorithm: perplexity, 20 (P_20); learning rate, 200 (E_200); iterations, (I_600); and theta, 0.5 (T_0.5). The Wilcoxon test was used to compare differences between time points. *P < .05; **P < .01; ***P < .001.

We also assessed the time-dependent changes in healthy controls and the differences between patients with allergy and healthy controls at each analyzed time point (see Figs E15, *C* and E16). We observed a higher frequency of pDCs in healthy controls when compared with individuals with allergy at baseline, whereas we saw the reverse picture at 12 months, which resulted from an increase in pDCs in the patients and a decrease in the healthy controls. The frequency of $CD1c^+$ mDCs was lower in the patients with allergy at the 3-, 6-, and 12-month time points when compared with their frequency in allergy-free individuals. The patients with allergy presented a significantly higher frequency of $CD141^+$ mDCs at 6 and 12 months after initiation of AIT when compared with the frequency in allergy-free individuals at the same time points.

tSNE analysis demonstrated that both in the patients with allergy and in the controls, $CD1c^+$ mDCs were phenotypically heterogeneous at baseline (Fig 6, *E* and *F* and see Online Repository Fig E17 in this article's Online Repository at www.jacionline.org) and clustered into 3 separate populations. The complexity of $CD1c^+$ mDC clusters was associated with subtly different expression of CD1c and HLA-DR (see Fig E17). However, during AIT the complexity of $CD1c^+$ mDCs clusters decreased (Fig 6, *E*), whereas in some of the healthy controls it increased (Fig 6, *F*). In contrast, pDCs and $CD141^+$ mDCs formed only 1 cluster per subset at baseline and remained stable during AIT. A detailed description of the tSNE analysis of the DC clusters can be found in the Results section of the Online Repository (see Fig E17).

Overall, we found that AIT induced a substantial increase in pDCs, with no change in their composition. Moreover, we observed a decrease in the frequency of $CD1c^+$ mDCs during AIT. Furthermore, we found a slight increase in $CD141^+$ mDCs in late time points of AIT. Finally, the heterogeneity of DCs assessed by tSNE analysis seems to be less complex than that of ILCs and monocytes. It all suggests that AIT-induced changes in the distribution of DC subsets increases their regulatory potential.[43]

DISCUSSION

The mechanisms of immune tolerance to allergens have been studied in humans by using various models, including AIT. Recent advances in understanding of the mechanisms of trained immunity in ILCs,[5] monocytes/macrophages,[44] and DCs,[44,45] as well as regulatory functions of ILCs or DCs,[46,47] strongly suggest that changes in these compartments should be studied in more detail in regard to induction of immune tolerance. In the present study, we demonstrated the results of 1 year of follow-up of circulating innate immune cells at 4 time points during the preseasonal AIT in comparison with the results for healthy controls at the same time points. Moreover, we extended the follow-up and assessed the composition of different innate immune subsets at the third-year time points, analyzing the stability of observed alterations. We showed that subcutaneous AIT for aeroallergens caused dynamic changes in the systemic innate immune response; in particular, we showed changes associated with the increased immunoregulatory phenotypes of innate immune cells. Thus, the observed shifts may underlie mechanisms facilitating trained tolerance to allergens.

Successful AIT is defined as a treatment that causes a significant reduction in allergic symptoms and the need for medications, with a concomitant decrease in proinflammatory cells and a subsequent increase in cell populations with anti-inflammatory or regulatory properties. These phenomena were often observed by our group and others in allergen-specific T-cell and B-cell compartments.[48-51] Notably, type 2 inflammation is a hallmark of allergic disease and is regulated by T_H2 effector cells, releasing related cytokines (including IL-4, IL-5, IL-9, and IL-13).[52] In addition, a growing body of evidence shows that ILCs, namely, ILC2s, support type 2 inflammation as an early source of IL-5 and IL-13 in response to epithelial-derived molecules, such as IL-33 and thymic stromal lymphopoietin.[7,53] Therefore, ILC2s are considered to reflect effector T_H2 cells in their cytokine expression pattern.[53] More importantly, allergen-trained (experienced) ILC2s were shown to persist long after resolution of inflammation and respond to the repeated allergen exposure more potently than untrained "naive" ILC2s did. Thus, they mediate more severe and long-term memory-like response to allergens.[54] Although the effector functions of ILC2s are associated with local inflammation, their rapid induction has been shown on the periphery after allergen challenge,[55] suggesting their trained immunity. Therefore, it is possible that circulating ILCs play an important role in regulation of allergic inflammation.[18] This explains the observed higher frequency of circulating ILC2s in individuals with allergy at baseline and in the pollen season that were reduced by AIT and finally reached the levels observed in healthy controls. These changes may result from the AIT-mediated trained tolerance, and they remain stable up to third year after AIT.[56,57] Our findings support previous reports showing significant decrease in ILC2s during the course of subcutaneous AIT,[17,19] with the novel findings about the relative stability of their intrasubset clusters. ILC2s possess phenotypic heterogeneity and functional plasticity,[8-10,58] which has not been previously studied in the context of AIT. Recently, $c\text{-}Kit^+$ ILC2s were shown to retain an ILC3-like phenotype and the ability for the conversion to IL-17-producing ILC3s.[59,60] On the other hand, however, a $c\text{-}Kit^{-/low}$ subset has been proposed to represent fully matured and lineage-committed ILC2 subset with high expression of GATA3 and high secretion of type 2 cytokines.[60] In our study, the vast majority of ILC2s were $c\text{-}Kit^-$; however, we also detected a small ILC2 cluster with low c-Kit expression in both allergic and allergy-free individuals. Interestingly, the $c\text{-}Kit^{low}$ cluster was not presented as a separate peak in the grass pollen season in both allergic and allergy-free individuals. It seems that AIT may not only reduce the frequency of ILC2s but may also limit their heterogeneity during the pollen season. However, our understanding of ILC2 plasticity in patients with allergy within the season needs to be further evaluated. Similarly, to date, the role of circulating ILC1s and ILC3s in allergic inflammation has remained elusive and needs more attention. In this study, we found that the frequency and heterogeneity of ILC1s and ILC3s changed after AIT. Within the set of ILC1s, we found an expansion of a novel cluster of $CD25^{++}$ ILC1s. Furthermore, we observed the emergence of a novel cluster of $CD127^+CD25^{++}c\text{-}Kit^+$ ILC3s during therapy. The appearance of $CD25^{++}$ ILC1 clusters may indicate acquisition of a regulatory phenotype, as shown previously for ILC2s.[47] NK cells do not seem to play a significant role in AIT because no differences in the frequency of either $CD56^{++}CD16^{+/-}$ or $CD56^+CD16^{++}$ subsets were observed.

Until recently, only a limited number of studies in humans have referred to the changes in the phenotype of antigen-presenting

cells. It was previously demonstrated that AIT induces a tolerogenic phenotype of DCs, which consequently may lead to the induction of regulatory T cells and a reduction of T_H2 cells.[61] Notably, here we are showing that this process may be supported by shifts in the particular subpopulations of monocytes, namely, an increase in the intermediate monocytes and decrease in the nonclassical monocytes. In addition, we noted an AIT-induced increase in the monocyte clusters with low HLA-DR expression. High HLA-DR expression level is associated with monocyte activation and their proinflammatory function.[62,63] Taken together, therefore, the observed changes may be recognized as an AIT-induced decrease in the activation of circulating monocytes. Interestingly, monocytes with proinflammatory properties, which have a nonclassical, activated phenotype ($CD14^+CD16^{++}$) recruited from the periphery, are crucial players in the promotion of local inflammatory responses in individuals with allergy.[64] In contrast, intermediate monocytes were shown to possess anti-inflammatory properties with significantly reduced expression of proinflammatory TNF and IL-1β and high expression of anti-inflammatory IL-10.[31] Moreover, they have reduced capability to present antigens to naive T cells in lymph nodes and thus may serve as tolerogenic cells.[65] Consequently, our current findings that the AIT-induced shift toward the increase in intermediate monocytes and decrease in nonclassical monocytes, together with the decrease in $HLA\text{-}DR^+$ clusters, may indicate the systemic response to limit local inflammation and support locally induced tolerance to allergens.

Notably, tolerogenic DCs represent a heterogeneous subset of cells with immature or semimature phenotype that is highly sensitive to local microenvironmental changes. After stimulation with inflammatory mediators and antigens, the maturation process is induced. It manifests itself in changes in both the phenotype of DCs and the composition of different DC subsets.[66] We have demonstrated that this process might be observed in the pollen season in both allergy-free individuals and AIT-treated individuals with allergy. Notably, pDCs in their steady state express low levels of HLA-DR and costimulatory molecules[67] and induce suboptimal T-cell priming, leading to T-cell anergy or tolerance. They have been shown to induce regulatory T cells and mediate oral tolerance.[43,68] Furthermore, pDCs may exert a protective role against type 2 inflammation.[69,70] Therefore, the lower frequency of circulating pDCs in individuals with allergy at baseline may reflect their lower tolerogenic potential, which is further targeted and efficiently restored by AIT as a manifestation of trained tolerance. Our findings are in line with previous observations showing an increased frequency of pDCs after 1 year of AIT.[71] In contrast to our current results, however, no changes in any mDCs subsets after 8 weeks or 1 year of subcutaneous AIT were observed.[71] This might be due to the slightly different time points and differences in the AIT schemes. It should be noted that mDCs were shown to support T_H2 responses in patients with allergy and thus may promote allergic inflammation.[72] Therefore, the systemic reduction in the frequency of $CD1c^+$ mDCs during the course of AIT that was observed in this study may be associated with the previously reported reduction of T_H2 cells.

In summary, here we have shown that AIT induces time-dependent changes in the composition and phenotypes of circulating ILCs, monocyte, and DC subpopulations. Our data demonstrate an *in vivo* switch toward tolerance-inducing subsets with a subsequent reduction of proinflammatory cells. It represents evidence of an AIT-induced trained tolerance and underlines the need for complex assessment of circulating innate immune cells, which may open a new window for monitoring AIT responses.

Key messages

- **ILC2s and ILC3s are downregulated and ILC1s are upregulated during the course of AIT.**
- **The AIT-induced systemic decrease of ILC2s and increase of ILC1s remains stable up to the third year of AIT.**
- **ILC1 clusters with higher CD25 expression are increased during the course of AIT.**
- **AIT induces a shift from a proinflammatory (nonclassical) toward an anti-inflammatory (intermediate) phenotype of circulating monocytes in the first year after AIT.**
- **pDCs and $CD141^+$ mDCs are upregulated during the course of AIT.**
- **AIT induces changes in the repertoire of systemic innate immune cells, restoring their frequency and composition toward a healthy immune status.**

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