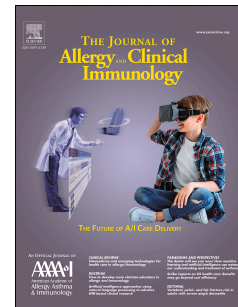


Journal Pre-proof

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

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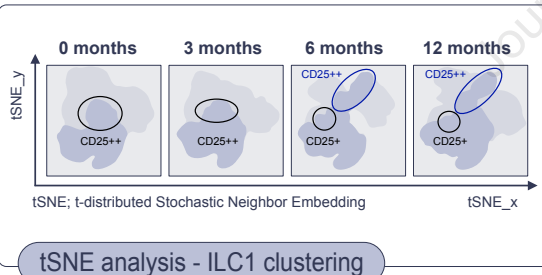
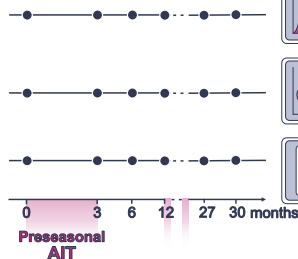
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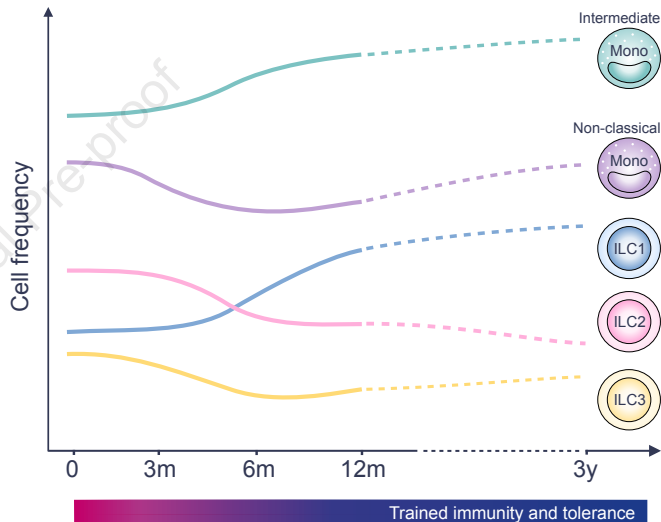


Trained immunity and tolerance in innate lymphoid cells, monocytes, and dendritic cells during allergen-specific immunotherapy

Study design



AIT-induced immune changes



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

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Abstract

Background: Despite efficacy of allergen-specific immunotherapy (AIT), the role of trained immunity and tolerance in this process has not been elucidated.

Objective: Here, we performed a comprehensive, longitudinal analysis of systemic innate immune cell repertoire in the course of AIT.

Methods: Allergic patients received standard preseasonal subcutaneous AIT with allergoids to birch and/or grass. Healthy controls were monitored without any intervention. Flow cytometry of ILC, NK, monocyte, and DC subsets was performed at baseline, 3 months (birch season), 6 months (grass seasons), and 12 months after the therapy in patients or in similar seasonal time points in controls. Additional analyses were performed in the 3rd-year birch and grass season.

Results: We observed a durable decrease of ILC2 and an increase of ILC1 after AIT with dynamic changes in their composition. We found that an expansion of CD127⁺CD25⁺⁺ clusters caused observed shifts in ILC1 heterogeneity. In addition, we observed development of CD127⁺CD25⁺⁺c-Kit⁺ ILC3 clusters. Moreover, we found an increase in intermediate monocytes, in parallel to the reduction of non-classical monocytes during first year after AIT. Classical and intermediate monocytes presented significant heterogeneity in allergic patients, but AIT reduced HLA-DR⁺⁺ clusters. Finally, an increase in pDCs and CD141⁺ mDCs was observed in allergic individuals, while CD1c⁺ mDCs were reduced during 1st 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 ILC, monocytes, and DCs during AIT might serve as a novel biomarker strategy.

Key Messages

- ILC2 and ILC3 are downregulated and ILC1 upregulated in the course of AIT
- AIT-induced systemic decrease of ILC2 and increase of ILC1 remains stable up to 3rd year of AIT
- ILC1 clusters with higher CD25 expression are increased in the course of AIT
- AIT induces shift from proinflammatory (non-classical) towards anti-inflammatory (intermediate) phenotype of circulating monocytes in the 1st year after AIT
- pDCs and CD141⁺ mDCs are upregulated in the course of AIT
- AIT induces changes in the repertoire of systemic innate immune cells, restoring their frequency and composition towards a healthy immune status

Capsule Summary

AIT induces time-dependent changes in the systemic composition of subpopulations of ILC, monocytes and DCs with increased regulatory and anti-inflammatory potential.

Key words

allergen immunotherapy, innate immune cells, antigen presenting cells, monocytes, DCs, ILC, NK cells

Abbreviations

AIT (Allergen-Specific Immunotherapy)

CD (Cluster of Differentiation)

DCs (Dendritic Cells)

ILC (Innate Lymphoid Cells)

IL (Interleukin)

LPS (Lipopolysaccharide)

CD1c⁺ mDCs (myeloid CD1c⁺ Dendritic Cells)

CD141⁺ mDCs (myeloid CD141⁺ Dendritic Cells)

NK cells (Natural Killer Cells)

pDCs (Plasmacytoid Dendritic Cells)

- 98 PBMCs (Peripheral Blood Mononuclear Cells)
- 99 sIgE (Specific Immunoglobulin E)
- 100 sIgG4 (Specific Immunoglobulin G4)
- 101 tIgE (Total IgE)
- 102 TSLP (Thymic Stromal Lymphopoietin)
- 103 tSNE (T-distributed Stochastic Neighbor Embedding)
- 104
- 105

Introduction

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 and 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, while innate immune mechanisms still remain elusive¹. Innate immune cells such as innate lymphoid cells (ILC), cytotoxic innate lymphoid cells (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 more (trained immunity) or less (trained tolerance) intense response towards the second challenge, and is referred to as innate immune memory⁴⁻⁶. 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 in the course of AIT will help to fully elucidate complex mechanisms providing long-lasting tolerance to allergens.

Based on the specific patterns of cytokine production, membrane markers and transcription factor expression, ILC are classified into three functionally distinct subsets, namely: group 1 (ILC1), group 2 (ILC2), and group 3 (ILC3) innate lymphoid cells, resembling Th1, Th2 and Th17 subsets, respectively⁷. In addition, due to the current advances in single cell sequencing, even greater heterogeneity of tissue and peripheral blood ILC has been reported⁸⁻¹⁰. ILC play a crucial function in the early stage of antimicrobial response, tissue repair and preservation of epithelial integrity¹¹⁻¹⁴. ILC2 have essential functions in allergic diseases. Increased numbers of these cells are observed in peripheral blood after allergen challenge and they are regulated during AIT¹⁵⁻¹⁹. Thus, the role of ILC in allergic inflammation, as well as their phenotypical and functional heterogeneity need further evaluation.

Similar to ILC, 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 based on their surface markers expression, namely CD56⁺CD16⁺⁺ and CD56⁺⁺CD16^{+/low} NK cells. Notably, CD56⁺ NK cells represent the predominant subset (up to 90%) on the periphery, and act mainly as cytotoxic cells and produce low levels of cytokines, while CD56⁺⁺ NK cells are recognized as cytokine producers²⁰. Interestingly, NK cells were

reported to be activated by allergen extracts in a non-specific manner and they can promote type-2 immune response and eosinophilic inflammation²¹.

Monocytes and DCs are antigen presenting cells and 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 profiles²²⁻²⁴. In fact, they have been proposed as crucial mediators of immunological tolerance²⁵⁻²⁷, also in response to allergens in allergic rhinitis²⁸ and in fatal asthma in children²⁹.

Monocytes represent a heterogeneous cell population in terms of phenotype and biological properties. According to the differences in CD14 (LPS receptor) and CD16 (Fcγ receptor III) expression, three functionally distinct subsets exist in humans, namely classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺) and non-classical (CD14⁺CD16⁺⁺) monocytes. Non-classical monocytes are considered as proinflammatory cells that upon stimulation release high amounts of proinflammatory cytokines, including TNF, and possess high cytotoxic and phagocytic activities. Elevated frequency of these cells are observed in proinflammatory diseases, infections, and sepsis³⁰. In contrast, intermediate monocytes have been referred to as anti-inflammatory since upon stimulation they constitute the principal source of IL-10 among all three subsets^{31, 32} and 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 as a more mature form of monocytes, when compared to the classical subset, which can next differentiate into monocyte-derived DCs^{31, 35}.

It is generally accepted that three distinct DC subsets can be distinguished in peripheral blood in humans, namely plasmacytoid DCs (CD303⁺ pDCs), myeloid CD1c⁺ mDCs and myeloid CD141⁺ mDCs, with additional extent of heterogeneity being discovered due to single cell sequencing^{32, 36}. pDCs are considered as an immature form of DCs since after a contact with antigen, which acts as a maturation signal, can present antigen to the T cells immediately in the lymph nodes. The remaining two subsets of mDCs show features of both immature DCs and CD303⁺ DC precursors³². Interestingly, it has been previously postulated that mDCs are involved mainly in the development of Th2 responses, whereas pDCs may induce anti-viral responses or orchestrate tolerance development by induction of regulatory T cells³⁷⁻³⁹.

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

Methods

Patients and healthy controls

Grass and/or birch pollen allergic subjects and healthy nonallergic, nonatopic controls were recruited in spring/summer 2015 at Inselspital Bern (Bern, Switzerland). The study was initiated in December 2015/January 2016, when patients received preseasonal subcutaneous immunotherapy to birch and/or grass. All subjects lived in the region of Bern (Switzerland). Patients were clinically diagnosed with pollen allergic rhinitis (symptoms in birch and/or grass season, positive skin prick tests and the presence of allergen-specific IgE (sIgE), assessed with Immunocap (Phadia AB, Uppsala, Sweden). Some patients had concomitant well-controlled allergic asthma⁴⁰. Patients did not suffer from any other clinically significant concomitant acute or chronic diseases. Further exclusion criteria were pregnancy, non-compliance, inability to follow the study procedures, drug or alcohol abuse, or participation in a study involving investigational drug use one month prior and during the course of this study. Twelve nonatopic, nonallergic healthy control subjects from the same region as allergic patients were followed in the similar seasonal time points as the patient's population to control for potential seasonal changes in the innate immune cell populations. Detailed demographic and clinical characteristics of study participants is shown in Table 1. 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 expressed written consent.

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

All allergic patients started the first course of subcutaneous preseasonal AIT in between the end of November 2015 to the beginning of January 2016, and then in the same format in the following two years (Online Repository Fig. E1A). 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) (Online Repository Fig. E1B-C). The standard therapy protocol consisted of increasing concentrations with a total of 7 – 9 injections (Online Repository Fig. E1B-C), given within

first 2-3 months. For patients receiving both tree and grass pollen AIT, preparations were injected separately each in opposite arms. Birch and grass pollen counts were retrieved from the Federal Office of Meteorology and Climatology MeteoSwiss (Zurich-Airport, Switzerland) (Online Repository Figure E1D-E). Blood samples were collected at four time points: time point 0 (immediately before the start of preseasonal AIT), after 3 months (at the end of preseasonal therapy; peak of birch pollen season), after 6 months (peak of grass pollen season), and at 12 months (out of season) for patients and in similar time points for healthy controls (Online repository Fig. E1)⁴¹. In 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 (SIAF, 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, peripheral blood mononuclear cells (PBMCs) 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) Guidelines⁴². Combined symptom and medication scores (CSMS) were recorded by the CSMS questionnaires in the doctors' office in the season before, and at each study visit, together with sampling. Non-responders to AIT were defined as those subjects who had 30% or less improvement in CSMS as compared to the season preceding initiation of AIT. Total IgE (tIgE), specific IgE (sIgE) to Phl p 1 combined with Phl p 5b, sIgE to Bet v 1 antibodies were measured in plasma using Immucap (Phadia AB, Uppsala, Sweden).

Flow cytometry

For ILC analysis, 20 million freshly isolated PBMCs were stained with a panel of anti-human antibodies. All antibodies with their clones and manufactures are presented in the Online Repository Table E1. ILC were characterized as viable, lineage negative (CD3⁻CD4⁻CD19⁻CD14⁻CD16⁻CD1a⁻CD11c⁻CD34⁻CD94⁻CD123⁻CD303⁻FcεRIa⁻) CD127⁺ cells and further discriminated as CRTH2⁺ ILC2, CRTH2⁻c-Kit⁻ ILC1, CRTH2⁻c-Kit⁺NKp44⁻ ILC3 (applied gating strategy is presented on the Online Repository Fig. E2).

For immunophenotyping of monocytes, DCs and NK cells 200 uL of fresh whole blood was stained with panel of anti-human antibodies according to stain-lyse-wash protocol. Briefly, antibodies were added to 200 uL of whole blood samples, mixed and incubated for 30

min 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 PBS and analyzed by using flow cytometry. Monocytes were classified based on morphology and fluorescent markers (viable CD3⁻CD19⁻CD14⁺). Subpopulations were identified as classical, intermediate and non-classical monocytes by their expression of CD14⁺⁺CD16⁻, CD14⁺⁺CD16⁺ or CD14⁺CD16⁺⁺, respectively (applied gating strategy is presented on the Online Repository Fig. E3). DC subsets were classified as viable CD3⁻CD19⁻HLA-DR⁺ cells and specific marker expression, namely CD123⁺⁺CD303⁺ for pDCs, HLA-DR⁺⁺CD1c⁺ for CD1c⁺ mDCs and CD14⁻CD141⁺⁺ for CD141⁺ mDCs (applied gating strategy is presented on the Online Repository Fig. E4). NK cells were classified as viable CD19⁻CD4⁻CD14⁻CD56^{+/++}CD16^{+/++} cells (applied gating strategy is presented on the Online Repository Fig. E5).

All specimens were acquired by using FACS ARIA III flow cytometer followed by data analysis in FlowJo 10.1 (TreeStar, Ashland, OR, USA). Classical gating and t-distributed Stochastic Neighbor Embedding (tSNE) algorithms were used to assess the subpopulations. Frequency of each analyzed subpopulation was calculated in the parent population in gating, unless specified otherwise. Total cell number was recalculated per uL of whole blood, based on the initial values measured by Sysmex cell counter.

For two-dimensional reduction of manual gates FlowJo tSNE plugin 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 particular gate available in all time points. Next, all files were concatenated in to new datafile and tSNE algorithm was applied. Following settings were used for tSNE algorithm perplexity 20 (P_20), learning rate 200 (E_200), iterations 600 (I_600), Theta 0.5 (T_0.5).

Statistics

Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software, La Jolla, United States). Categorical variables were analyzed with the Fisher exact test, while continuous variables with 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<0.05.

Results

Group 2 and group 3 innate lymphoid cells decrease during the course of AIT

First, we investigated the effect of AIT on the frequency and absolute numbers of three main and best characterized subsets of ILC, namely ILC1, ILC2 and ILC3 (Fig. 1A). Three types of comparisons were made i) 1st-year time points compared to baseline, ii) 3rd-year after initiation of AIT compared to the 1st-year time points, and iii) allergic patients compared to the healthy controls analyzed at the same time points. We found increased frequency and numbers of ILC1 at 6- and 12-month time points (Fig. 1B and Online Repository Fig. E6A). In contrast, the frequency of ILC2 was decreased after 12 months. Interestingly, the frequency of ILC2 decreased most between 6- and 12-months' time point; however, only a trend can be observed in ILC2 cell numbers (Fig. 1C and Online Repository Fig. E6A). ILC3 frequency also decreased after 12 months (Fig. 1D).

Importantly, we noticed that observed in the 1st-year effects of AIT on ILC subsets remained stable or were even more profound in the 3rd-year (Online Repository Fig. E6B). In detail, we observed a further increase in the frequency of ILC1 and a further decrease in the frequency of ILC2. We found no changes in the frequency of ILC3 between 1st- and 3rd-year of AIT in the birch pollen season, while the frequency of ILC3 in the grass pollen season was increased (6- vs. 30-month time point).

Having found the time course changes in ILC subsets during the 1st- year after AIT, we evaluated the differences between the frequency of analyzed cells in allergic individuals in the course of AIT and matched controls in the same seasonal time points. Healthy controls showed no seasonal changes of all analyzed ILC subsets (Online Repository Fig. E6C). In comparison of controls to patients at the same time points, a lower frequency of ILC1 in allergic patients at baseline and 6 months after initial treatment was observed (Fig. 1E). After 12 months of treatment, there was no difference in the ILC1 frequency between patients and controls. Elevated frequency of ILC2 in allergic donors 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. 1F). There were no differences in the frequency of ILC3 between patients and controls in all analyzed seasonal time points (Fig. 1G).

Next, we used tSNE algorithm to analyze heterogeneity of ILC subsets clustering at baseline and their changes over time (Fig. 2). As expected, analyzed ILC subpopulations clustered separately confirming the use of correct gating strategy (Online Repository Fig. E2, tSNE ILC subsets). In-depth analysis of cluster heterogeneity with subsequent quantification of newly identified subsets by classical gating, we noticed intriguing changes in ILC1 and ILC3, but not ILC2 phenotypes over time. Briefly, AIT induced an increase in numbers of

ILC1 clusters with higher CD25 expression (Fig. 2A) and thus it made their signature comparable to nonallergic individuals at 12 months (Online Repository Fig. E7). In fact, there was a significant elevation of the frequency of CD127⁺CD25⁺⁺ cluster at the six-month time point (Fig. 2B). In contrast, the frequency of CD127⁺⁺CD25⁺⁺ subset decreased at 12 months when compared to baseline (Fig. 2C). Interestingly, we found that ILC2 were quite heterogenous, but there were no significant changes in the presence (Fig. 2D and Online Repository Fig. E8) or frequency of ILC2 clusters (Fig. 2E and F) over time. The signature of ILC3 subpopulations in allergic individuals was less complex at baseline (Fig. 2G) and differed significantly from controls (Online Repository Fig. E9). At 3 months and at 6 months after initiation of AIT, there was an increase in the complexity of ILC3 clusters, associated with the expansion of CD127⁺CD25⁺⁺c-Kit⁺ (Fig. 2H) and decrease of CD127⁺⁺CD25⁺⁺c-Kit⁺⁺ cells (Fig. 2I). Detailed description of ILC clusters can be found in the online repository results (Online Repository Fig. E7-E9).

Taken together, our data suggest that AIT limits circulating ILC2 and ILC3, in favor of an expansion of ILC1. In addition, AIT also induces an increase in ILC1 and ILC3 heterogeneity, bringing their phenotypes closer to 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 ILC, next we analyzed changes in the frequency and numbers of innate lymphoid cells with cytotoxic potential, namely CD56⁺CD16⁺⁺ (CD56 dim) and CD56⁺⁺CD16^{+/-} (CD56 bright) NK cells (Fig. 3A). We observed no changes in the frequency of NK cells in allergic patients undergoing AIT, mainly due to huge individual variations (Fig. 3B and C). Similarly, we found no significant changes in the numbers of both analyzed subsets (Online Repository Fig. E10A). As an interesting finding, which requires further investigation, nonallergic individuals showed a significant decrease in the frequencies of both CD56 bright and CD56 dim NK cells during the birch season (3 months, Online Repository Fig. E10B). Consequently, we found higher frequency of CD56⁺CD16⁺⁺ NK cells in allergic patients at 3-month time point, as compared to healthy controls, while no differences were observed at 6 and 12 months (Fig. 3D). Moreover, no differences in the frequency of CD56⁺⁺CD16^{+/-} NK cells, between AIT patients and healthy controls, in the same seasonal time points were observed (Fig. 3E).

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

Monocytes shift from proinflammatory towards anti-inflammatory phenotypes in 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. 4A). We observed a substantial decrease in the frequency of non-classical $CD14^+CD16^{++}$ monocytes already at 3 months (Fig. 4B). This decrease was even more profound at 6 months (Fig 4B and Online Repository Fig. E11A). Moreover, there was a steady increase in the intermediate $CD14^{++}CD16^+$ monocytes in the course of AIT, reaching maximum at 12 months (Fig. 4C and Online Repository Fig. E11A), while the frequency of classical $CD14^{++}CD16^-$ monocytes showed alterations reflecting changes in the other subpopulations (Fig. 4D and Online Repository Fig. E11A).

Notably, in contrast to ILC subsets, the effects of AIT on the composition of different monocyte subsets was less stable over time (Online Repository Fig. 11B). In contrast, to the early (1st-year) effects of AIT, we found a significant increase in the frequency of $CD14^+CD16^{++}$ non-classical monocytes in the 3rd-year pollen season. Simultaneously, the frequency of $CD14^{++}CD16^+$ intermediate monocytes did not increase at this time (Online Repository Fig. E11B).

Next, we compared the frequency of different monocyte subsets between allergic patients and healthy controls at the same time points. Interestingly, we found that there was slightly reduced frequency of non-classical monocytes at 12 months in patients (Fig. 4E). Surprisingly, no differences were observed in intermediate monocytes (Fig. 4F). However, we noticed slight, but significant differences in classical monocytes at 3 and 6 months (Fig. 4G and Online Repository Fig. E11C).

tSNE analysis of the different monocyte subsets demonstrated that all analyzed clusters separated from other cell types, confirming their distinct phenotype (Online Repository Fig. E3, tSNE APC subsets). We found no change in the complexity of non-classical monocytes in the AIT patients (Fig. 5A) and healthy controls (Online Repository Fig. E12), yet there was an increase in the expression of HLA-DR (Fig. 5B) with stable

expression of CD141 in this subset (Fig. 5C). In contrast, we noticed significant heterogeneity in remaining two subsets, namely intermediate and classical monocytes. Briefly, we observed a reduction of HLA-DR⁺⁺ clusters in the intermediate monocytes (Fig. 5D and Online Repository Fig. E13), and further confirmed it by quantification of HLA-DR (Fig. 5E) and CD141 expression (Fig. 5F). In classical monocytes, we found an expansion of HLA-DR⁺CD141^{+/-} clusters at 12 months in allergic patients (Fig. 5G and Online Repository Fig. E14). Consequently, there was a slight decrease of HLA-DR expression from 3 months to 12 months (Fig. 5H) and a significant decrease of CD141 expression from 6 months to 12 months (Fig. 5I). More detailed description of different monocyte clusters can be found in the online repository results (Online Repository Fig. E12-14).

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

CD1c⁺ mDCs, but not CD141⁺ mDCs and pDCs are downregulated in 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 different monocyte subsets, showing varying expression levels of CD14 and CD16, peripheral blood DCs have been characterized as more distinct subpopulations expressing characteristic pattern of membrane molecules (Fig. 6A). Here, we observed an increase in the frequency of pDCs at 6- and 12-month time points compared to 3 months (Fig. 6B). In addition, we found a substantial decrease in the frequency and numbers of CD1c⁺ mDCs in the course of AIT when compared to baseline (Fig. 6C and Online Repository Fig. E15A). Similarly, to non-classical monocytes, CD1c⁺ mDCs decreased significantly after 3 months of AIT and even further after 6 months (Fig. 6C). In addition, we observed a slight elevation of the frequency of CD141⁺ mDCs at 12 months when compared to 3 months (Fig. 6D). However, no differences were observed in the numbers of CD141⁺ mDCs, mainly due to noticeable individual variations (Online Repository Fig. E15A).

Similarly, to monocytes, the AIT-induced changes in the composition of DCs in the pollen season of the 1st year seem to be modified in the following seasons. We found a lower frequency of pDCs in the 3rd-year birch pollen season compared to 3 months post AIT (Online Repository Fig. E15B). The frequency of CD1c⁺ mDCs in the 3rd-year birch and grass pollen seasons remains comparable to the levels observed in the 1st year post AIT. In contrast, the frequency of CD141⁺ mDCs decreased significantly in the 3rd-year birch pollen season and was slightly increased in the grass pollen season, but still remained lower than in the representative seasons of the 1st year.

We also assessed the time-dependent changes in healthy controls and the differences between allergic patients and healthy controls in each analyzed time point (Online Repository Fig. E15C and E16). We observed higher frequency of pDCs in healthy controls when compared to allergic individuals at baseline and a reversed picture at 12 months, which resulted from an increase of pDCs in patients and a decrease in healthy controls. Frequency of CD1c⁺ mDCs was lower in the allergic patients at 3-, 6- and 12- month time points when compared to nonallergic individuals. Allergic patients presented significantly higher frequency of CD141⁺ mDCs at 6 and 12 months after initiation of AIT when compared to nonallergic individuals at the same time points.

tSNE analysis demonstrated that in both allergic patients and controls, CD1c⁺ mDCs were phenotypically heterogeneous at baseline (Fig. 6E, F and Online Repository Fig. E17) and clustered into three separate populations. The complexity of CD1c⁺ mDC clusters was associated with subtly different expression of CD1c and HLA-DR (Online Repository Fig. E17). However, during AIT the complexity of CD1c⁺ mDCs clusters decreased (Fig. 6E), while in some of the healthy controls increased (Fig. 6F). In contrast, pDCs and CD141⁺ mDCs formed only one cluster per subset at baseline and remained stable during AIT. Detailed description of tSNE analysis of the DC clusters can be found in the online repository results (Online Repository Fig. E17).

Overall, we found that AIT induced a substantial increase of 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, DCs heterogeneity assessed by tSNE analysis seems to be less complex compared to ILC and monocytes. It all suggests that AIT-induced changes in the distribution of DCs subsets, increases their regulatory potential⁴³.

Discussion

Mechanisms of immune tolerance to allergens have been studied in humans by using various models, including AIT. Recent advances in understanding mechanisms of trained immunity in ILC⁵, monocytes/macrophages⁴⁴, and DCs^{44, 45}, as well as regulatory functions of ILC 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 one year, four time points follow up of circulating innate immune cells in the preseasonal AIT in comparison to the healthy controls at the same time points. Moreover, we extended follow up and assessed a composition of different innate immune subsets at the 3rd-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, particularly 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 significant reduction of 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 compartments⁴⁸⁻⁵¹. Notably, type 2 inflammation is a hallmark of allergic disease and is regulated by Th2 effector cells, releasing related cytokines, including IL-4, IL-5, IL-9, and IL-13⁵². In addition, a growing body of evidence shows that innate lymphoid cells, namely ILC2, 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 (TSLP)^{7, 53}. Therefore, ILC2 are considered to reflect effector Th2 cells in their cytokine expression pattern⁵³. More importantly, allergen-trained (experienced) ILC2 were shown to persist long after resolution of inflammation and respond more potently than untrained “naïve” ILC2 to the repeated allergen exposure. Thus, they mediate more severe and long-term memory-like response to allergens⁵⁴. Although the effector functions of ILC2 are associated with local inflammation, their rapid induction has been shown on the periphery after allergen challenge⁵⁵ suggesting their trained immunity. Therefore, it is possible that circulating ILC play an important role in regulation of allergic inflammation¹⁸. This explains observed here higher frequency of circulating ILC2 in allergic individuals at baseline, and in the pollen season, which are 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 3rd year after AIT^{56, 57}. Our findings support previous reports showing significant decrease of ILC2 in the course of subcutaneous AIT^{17, 19}, with the novel findings

about the relative stability of their intrasubset clusters. ILC2 possess phenotypical heterogeneity and functional plasticity^{8-10, 58}, which has not been previously studied in the context of AIT. Recently, c-Kit⁺ ILC2 were shown to retain an ILC3-like phenotype and the ability for the conversion to IL-17-producing ILC3^{59, 60}. On the other hand, however, a c-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 cytokines⁶⁰. In our study, the vast majority of ILC2 were c-Kit⁻; however, we also detected small ILC2 cluster with low c-Kit expression in both allergic and nonallergic individuals. Interestingly, the c-Kit^{low} cluster was not presented as a separate peak in the grass pollen season in both allergic and nonallergic individuals. It seems that AIT may not only reduce the frequency of ILC2 but also limit their heterogeneity during the pollen season. However, our understanding of ILC2 plasticity in allergic patients within the season needs to be further evaluated. Similarly, to date, the role of circulating ILC1 and ILC3 in allergic inflammation remains elusive and needs more attention. We found here that ILC1 and ILC3 frequency and heterogeneity changed after AIT. Within ILC1, we found an expansion of a novel cluster of CD25⁺⁺ ILC1. Furthermore, we observed the emergence of a novel cluster of CD127⁺CD25⁺⁺c-Kit⁺ ILC3 during therapy. Appearance of CD25⁺⁺ ILC1 clusters may indicate acquiring a regulatory phenotype, as shown previously for ILC2⁴⁷. NK cells do not seem to play a significant role in AIT, since no differences in the frequency of neither CD56⁺⁺CD16^{+/-} nor CD56⁺CD16⁺⁺ subsets were observed.

Until recently, 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 reduction of Th2 cells⁶¹. Notably, we are showing here that this process may be supported by shifts in the particular subpopulations of monocytes, namely an increase in the intermediate and a decrease in the non-classical 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}. Therefore, the observed changes, may be altogether recognized as the AIT-induced decrease in the activation of circulating monocytes. Interestingly, monocytes of proinflammatory properties, which have non-classical, activated phenotype (CD14⁺CD16⁺⁺), recruited from the periphery, are crucial players in the promotion of local inflammatory responses in allergic individuals⁶⁴. In contrast, intermediate monocytes were shown to possess anti-inflammatory properties with significantly reduced expression of proinflammatory TNF

and IL-1 β and high expression of anti-inflammatory IL-10³¹. Moreover, they have reduced capability to present antigens to naïve T cells in lymph nodes and thus may serve as tolerogenic cells⁶⁵. Consequently, our current findings that AIT-induced shift towards the increase in intermediate and the decrease in non-classical monocytes, together with the decrease in HLA-DR positive 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 semi-mature phenotype, highly sensitive to local microenvironmental changes. After stimulation with inflammatory mediators and antigens, maturation process is induced. It manifests itself in changes of both DCs phenotype and composition of different DC subsets⁶⁶. We demonstrated that this process might be observed in the pollen season in both nonallergic and AIT-treated allergic individuals. Notably, pDCs in their steady state express low levels of HLA-DR and costimulatory molecules⁶⁷ 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 protective role against type-2 inflammation^{69, 70}. Therefore, the lower frequency of circulating pDCs in allergic individuals at baseline may reflect their lower tolerogenic potential, further targeted and efficiently restored by AIT, as manifestation of trained tolerance. Our findings are in line with previous observations showing increased frequency of pDCs after one year of AIT⁷¹. However, in contrast to our current results, no changes in any mDCs subsets after eight weeks and one year of subcutaneous AIT were observed⁷¹. 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 Th2 responses in allergic patients and thus may promote allergic inflammation⁷². Therefore, systemic reduction in the frequency of CD1c⁺ mDCs in the course of AIT, observed in this study, may be associated with previously reported reduction of Th2 cells.

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

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Table 1. Demography and clinical characteristics of study participants

DEMOGRAPHY			
	Allergic patients	Healthy controls	P value
N	13	12	
Age [y]	27 (19-36)	35.5 (23-63)	0.0222
Sex, female/male	5/7	10/2	0.0894
Weight [kg]	77 (63-100)	66.5 (53-102)	0.0612
Height [cm]	172 (162-187)	169 (153-188)	0.2295
CLINICAL DATA			
Allergic rhinitis, yes/no	13/0	0/12	<0.0001
Asthma, yes/no	8/5	0/12	0.0016
SPT positive, yes/no	13/0	0/12	<0.0001
Total IgE [kU/l]	106 (28-274)	26 (10-73)	0.0003
Phl p 1 + Phl p 5 IgE [kU/l]	9.88 (1.59-68.9)	nd	<0.0001
Bet v 1 IgE [kU/l]	2.99 (0.1-36.4)	nd	0.0005
FEV1 [%]	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 non-responders to birch AIT*, N [%]	2 (15.4)	n/a	n/a
Clinical non-responders to grass AIT*, N [%]	3 (23.1)	n/a	n/a

729 Data are presented as median with range, exact numbers or percentage. Data were analyzed by Mann-Whitney
730 test and Fisher exact test, as appropriate. * Non-responders to AIT were assessed as 30% or less decrease in
731 combined symptoms and medication score (CSMS) as compared to the appropriate pollen season preceding
732 initiation of AIT. FEV1- forced expiratory volume in first second; nd – not determined; n/a – not applicable

733

Figure legends

Figure 1. AIT induce decrease in group 2 and 3 innate lymphoid cells

(A) Representative gating of ILC1, ILC2 and ILC3 among $\text{lin}^-\text{CD127}^+$ ILC. Summary of flow cytometry analyses of AIT-induced changes in frequency of (B) ILC1, (C) ILC2 and (D) ILC3 in allergic patients in the course of AIT (n=11; Wilcoxon test was used). Each ILC subset is shown as a percentage in $\text{lin}^-\text{CD127}^+$ gate. Comparison of seasonal changes of (E) ILC1, (F) ILC2 and (G) ILC3 between nonallergic healthy controls (n=12), and allergic patients (n=11) in the course of AIT. Wilcoxon test was used to compare differences between time points. Mann-Whitney U test was used to compare differences between controls and AIT patients. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Figure 2. AIT induce changes in the heterogeneity of circulating ILC

Representative tSNE two-dimensional plots showing changes in complexity and clustering of different circulating ILC subsets in each analyzed time point in allergic AIT patients. (A) ILC1 clustering and quantification of (B) $\text{CD127}^+\text{CD25}^{++}$ ILC1 and (C) $\text{CD127}^{++}\text{CD25}^{++}$ ILC1 clusters (n=10). (D) ILC2 clustering and quantification of (E) $\text{c-Kit}^+\text{CD25}^{++}$ ILC2 and (F) $\text{c-Kit}^-\text{CD25}^{++}$ ILC2 clusters (n=11). (G) ILC3 clustering and quantification of (H) $\text{CD127}^+\text{CD25}^{++}\text{c-Kit}^+$ and (I) $\text{CD127}^{++}\text{CD25}^{++}\text{c-Kit}^{++}$ ILC3 (n=11). For tSNE analysis, the cells were gated as described in Online Repository Figure E2 and a two-dimensional reduction was performed from lin-CD127^+ gate (total ILC) for NKp44, CCR2, CD25, CD127, c-Kit, and ILT3 expression. Following settings were applied for tSNE algorithm perplexity 20 (P_20), learning rate 200 (E_200), iterations 1000 (I_1000), Theta 0.5 (T_0.5). Graph overlays were performed to visualize changes in cluster complexity of different ILC subsets namely, (A) ILC1, (D) ILC2 and (G) ILC3. For novel subset quantification manual gating was applied. Wilcoxon test was used to compare differences between time points. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Figure 3. AIT does not affect the composition of circulating NK cells

(A) Representative gating for $\text{CD56}^{++}\text{CD16}^{+/-}$ and $\text{CD56}^+\text{CD16}^{++}$ NK cells. Summary of flow cytometry analyses of AIT-induced changes in frequency of (B) $\text{CD56}^+\text{CD16}^{++}$ NK and (C) $\text{CD56}^{++}\text{CD16}^{+/-}$ cells in allergic patients in the course of AIT (n=11). Comparison of seasonal changes of (D) $\text{CD56}^{++}\text{CD16}^{++}$ and (E) $\text{CD56}^+\text{CD16}^{+/-}$ NK cells between nonallergic healthy controls (n=12), and allergic patients in the course of AIT (n=11). W Wilcoxon test was used

to compare differences between time points. Mann-Whitney U test was used to compare differences between controls and AIT patients. ** $p < 0.01$

Figure 4. Monocyte shift from proinflammatory towards anti-inflammatory phenotypes in the course of AIT

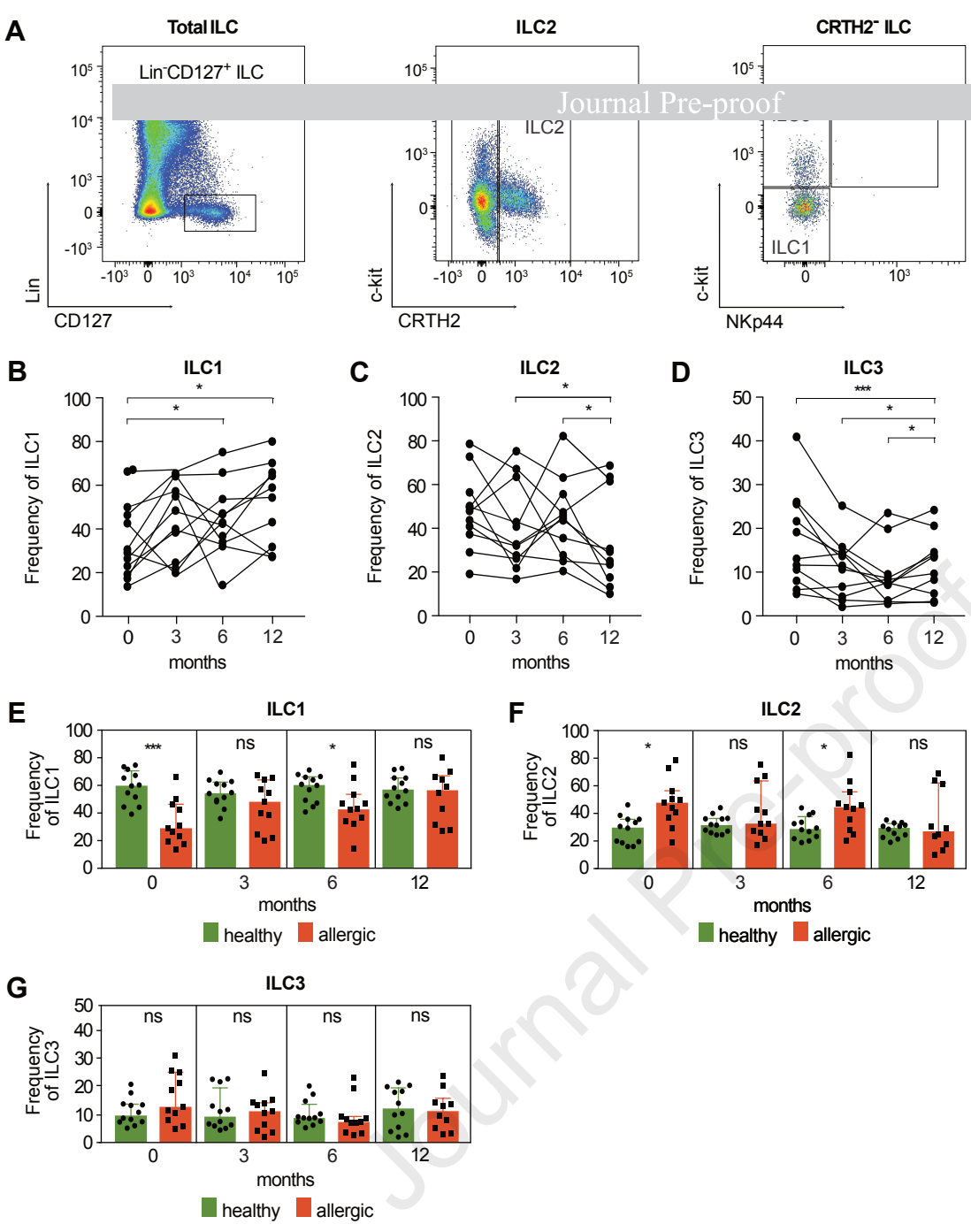
(A) Representative gating for $CD14^{++}CD16^{-}$ classical, $CD14^{++}CD16^{+}$ intermediate, and $CD14^{+}CD16^{++}$ non-classical monocytes and representative dot plots of different monocyte subsets in each analyzed time point. Summary of flow cytometry analyses of AIT-induced changes in the frequency of (B) $CD14^{+}CD16^{++}$ non-classical, (C) $CD14^{++}CD16^{+}$ intermediate, and (D) $CD14^{++}CD16^{-}$ classical monocyte subsets in allergic AIT patients ($n=12$). Comparison of seasonal changes of (E) $CD14^{+}CD16^{++}$ non-classical, (F) $CD14^{++}CD16^{+}$ intermediate, and $CD14^{++}CD16^{-}$ classical monocyte subsets between nonallergic healthy controls ($n=12$), and allergic patients ($n=12$) in the course of AIT. Wilcoxon test was used to compare differences between time points. Mann-Whitney U test was used to compare differences between controls and AIT patients. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns – not significant

Figure 5. AIT induce changes in the heterogeneity of different subsets of monocytes

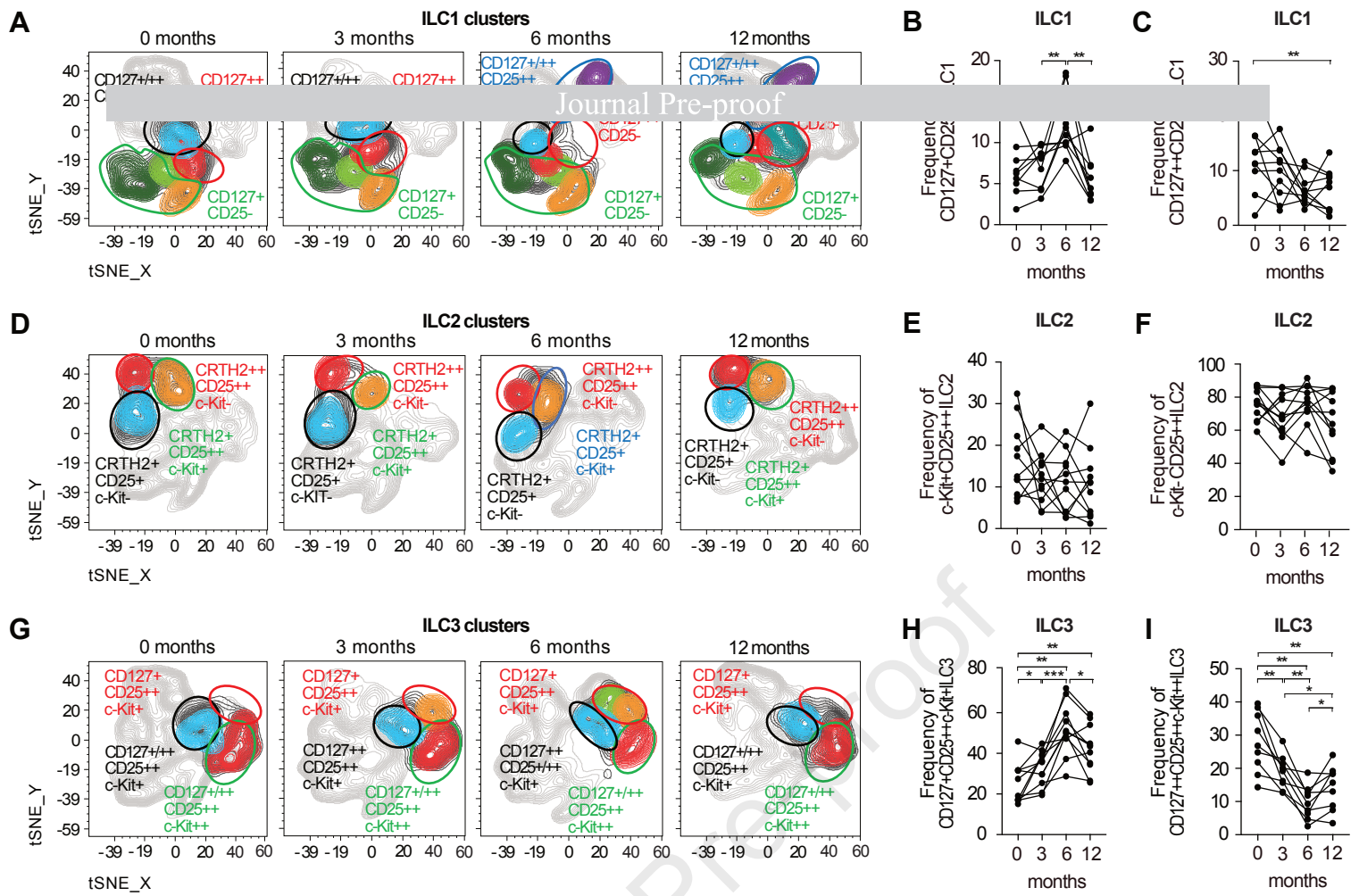
Representative tSNE two-dimensional plots representing changes in complexity and clustering of different monocytes subsets in each analyzed time point. For tSNE analysis, the cells were gated as follows. First flow check control was applied to gate out all disruptions in the instrument performance. Next doublet discrimination according to both forward scatter (FSC-H and FSC-A) and side scatter (SSC-H vs. SSC-W) was performed, followed by dead, CD3, and CD19 cell discrimination (viability). Next HLA-DR positive cells were gated. Finally, two-dimensional reduction was performed from $HLA-DR^{+}$ cells for CD1c, CD14, HLA-DR, CD16, CD123, CD141, and CD303 expression. Following settings were applied for tSNE algorithm perplexity 20 (P_20), learning rate 200 (E_200), iterations (I_600), Theta 0.5 (T_0.5). Graph overlays were performed to visualize changes in cluster complexity. (A) $CD14^{+}CD16^{++}$ non-classical monocytes clusters with quantification of (B) HLA-DR and (C) CD141 MFI. (D) $CD14^{++}CD16^{+}$ intermediate monocyte clusters with quantification of (E) HLA-DR and CD141 expression. (G) $CD14^{++}CD16^{-}$ classical monocyte clusters and quantification of (H) HLA-DR and (I) CD141 MFI ($n=11$). Wilcoxon test was used to compare differences between time points. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 6. pDCs and CD141⁺ mDCs are upregulated, whereas CD1c⁺ mDCs are downregulated in the course of AIT

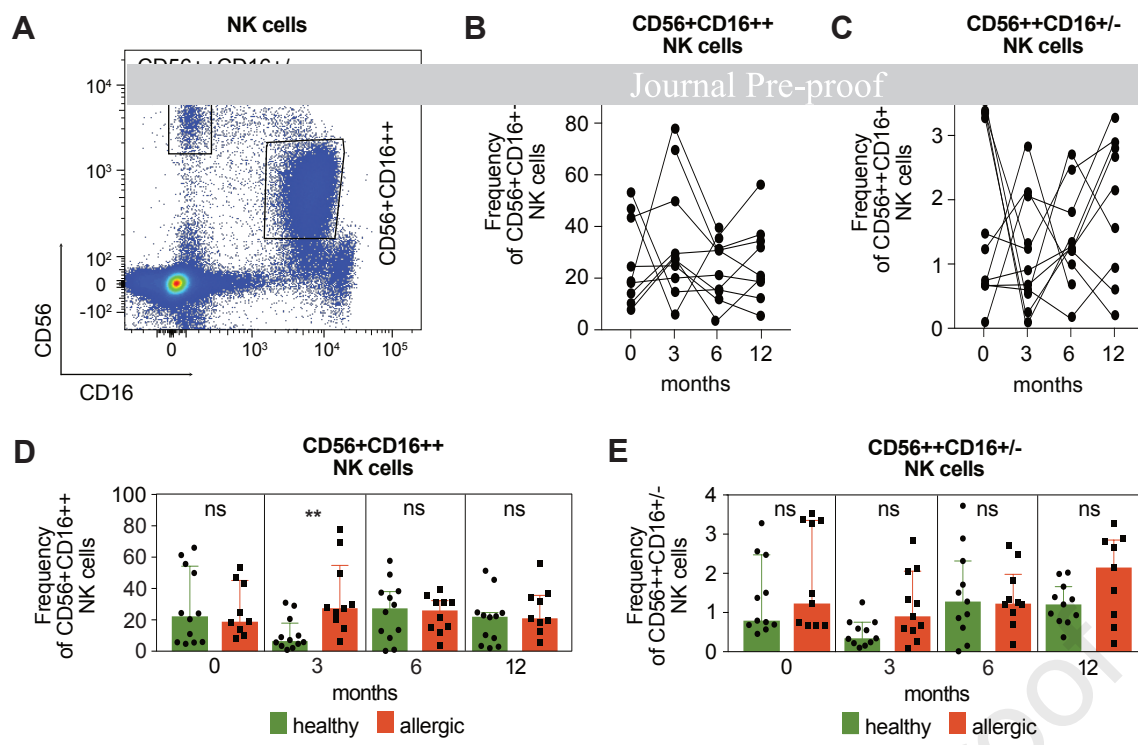
(A) Representative gating for pDCs, CD1c⁺ mDCs and CD141⁺ mDCs. Summary of flow cytometry analyses of AIT-induced changes in the frequency of **(B)** CD123⁺⁺CD303⁺ pDCs, **(C)** HLA-DR⁺⁺CD1c⁺ mDCs (CD1c⁺ mDCs), and **(D)** CD14⁻CD141⁺⁺ (CD141⁺ mDCs) in allergic patients in the course of AIT (n=12). Representative tSNE two-dimensional plots representing changes in complexity and clustering of different DC subsets in each analyzed time point in **(E)** allergic and **(F)** healthy nonallergic individuals. 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 according to both forward scatter (FSC-H and FSC-A) and side scatter (SSC-H vs. SSC-W) was performed, followed by dead, CD3, and CD19 cell discrimination (viability). Next, HLA-DR positive cells were gated. Finally, two-dimensional reduction was performed from HLA-DR⁺ cells for CD1c, CD14, HLA-DR, CD16, CD123, CD141, and CD303 expression. Following settings were applied for tSNE algorithm perplexity 20 (P_20), learning rate 200 (E_200), iterations (I_600), Theta 0.5 (T_0.5). Wilcoxon test was used to compare differences between time points. * p<0.05; ** p<0.01; *** p<0.001



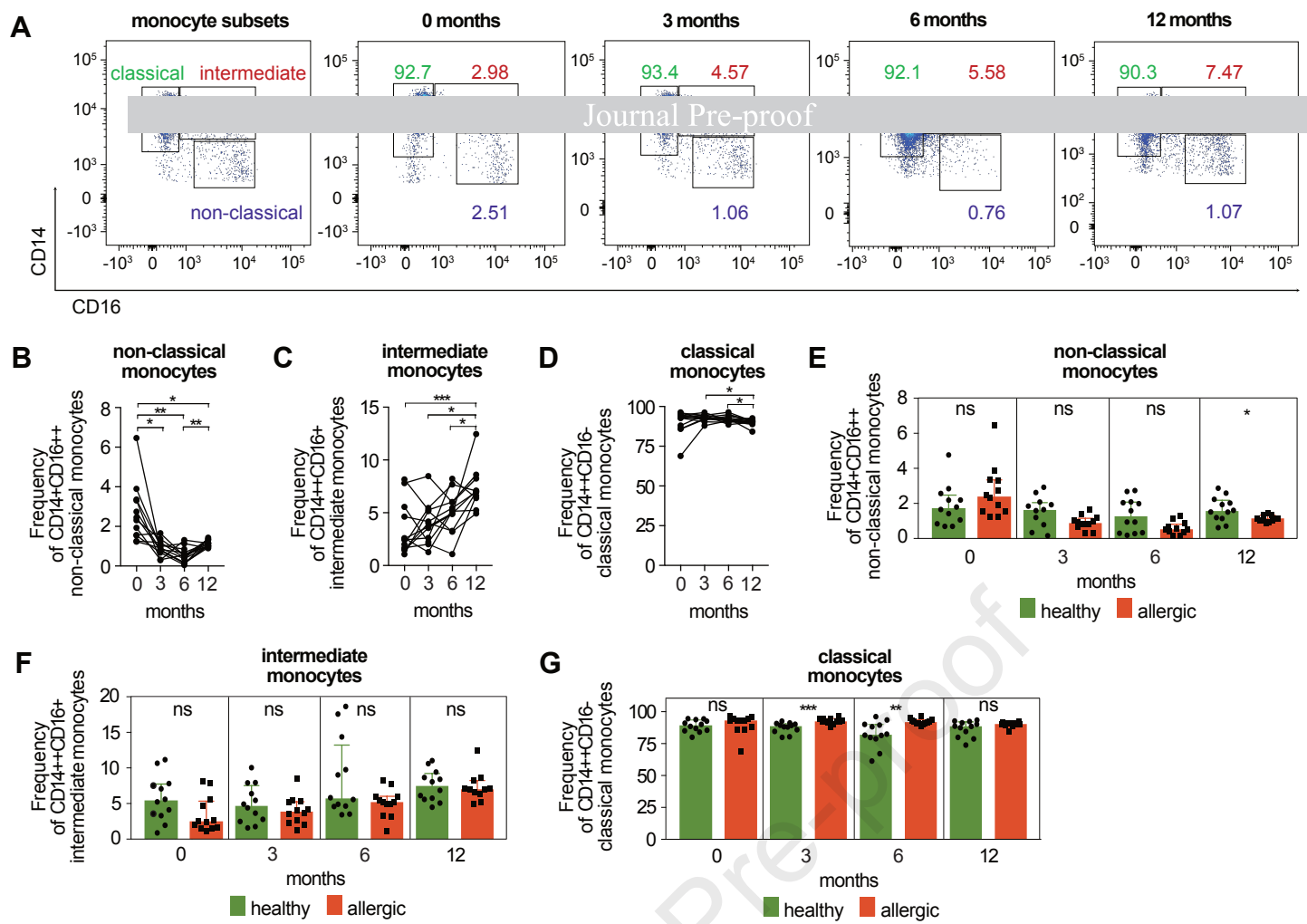
Eljaszewicz et al. Figure 1



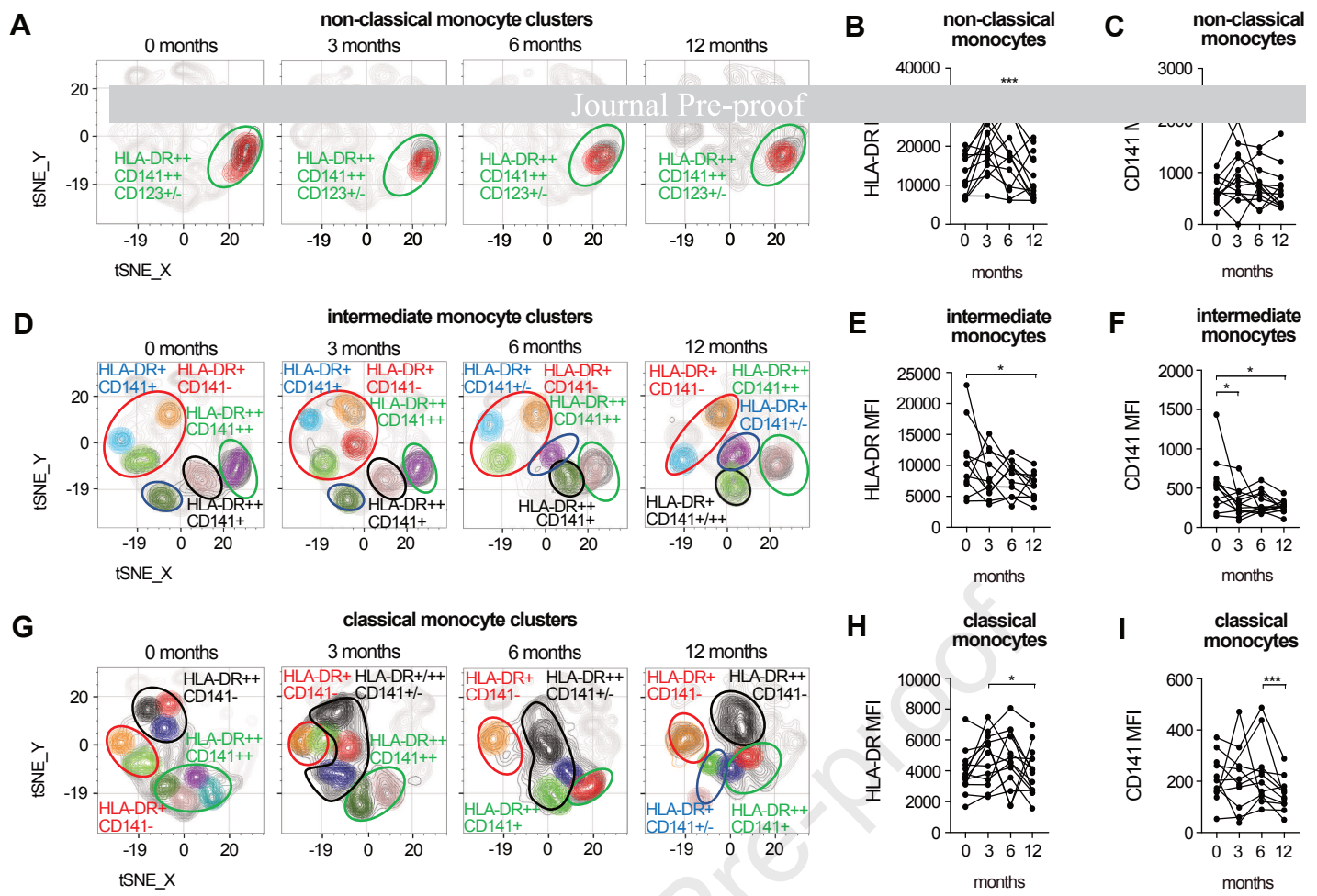
Eljaszewicz et al Figure 2



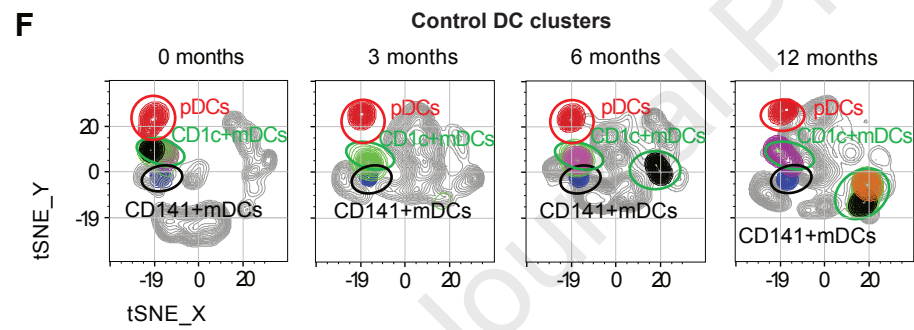
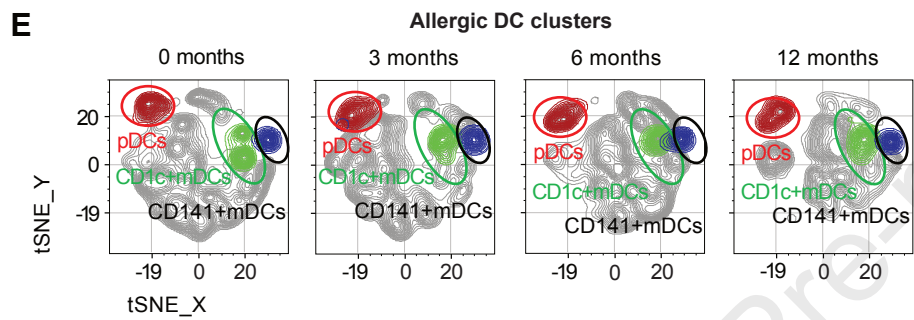
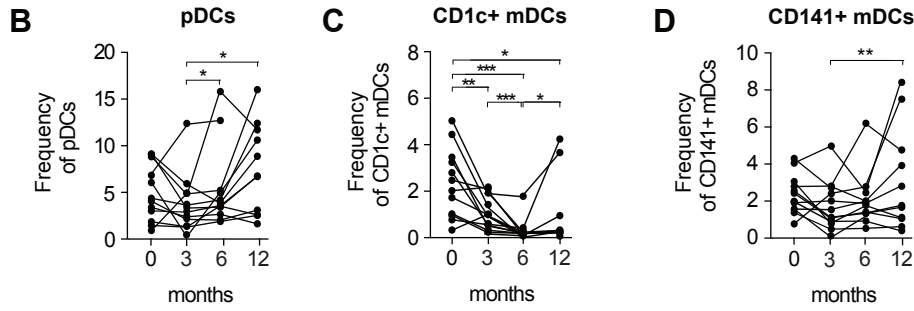
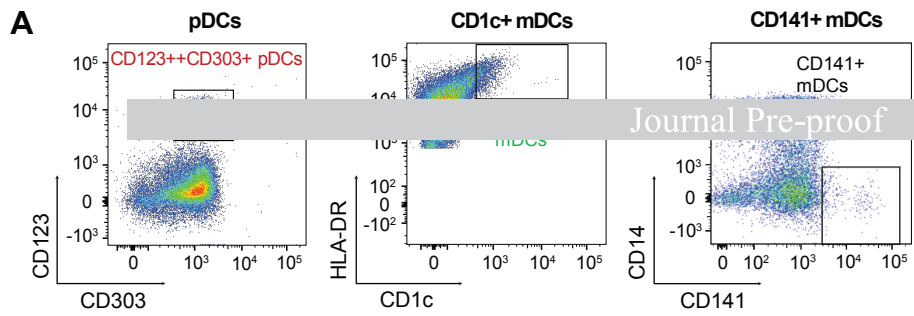
Eljaszewicz et al. Figure 3



Eljaszewicz et al Figure 4



Eljaszewicz et al Fig. 5



Eljaszewicz et al Figure 6