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Histology-Based Blood Leukocyte Profiling Reveals Parallel Th2 and Th17 Signatures in Seasonal Allergic Rhinitis

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Running head: Histology-based blood leukocyte profiling in allergic rhinitis

ABSTRACT

Background: Allergic rhinitis (AR) is one of the most common medical conditions in the westernized world. Recent data suggest AR to be far more immunologically complex than the archetypical allergic Th2-driven eosinophilic inflammation and new methodological approaches are needed to decode this complexity.

Methods: This study explores a novel histology-based analysis of circulating leukocytes for detailed profiling of immune cells using routine clinical blood samples. In brief, leukocytes were purified with minimal *ex-vivo* artefacts, embedded into agarose-paraffin pellets and sectioned for cutting-edge immunohistochemistry-based immune cell profiling. Blood leukocyte mapping was performed in 16 patients with seasonal AR outside and during the birch pollen season.

Results: Our methodological feasibility test confirmed that the > 5000 cross sectioned leukocytes typically present in a pellet section had well preserved morphology and cell marker epitopes, allowing for robust quantitative analysis of immune-stained slides. Blood leukocyte samples collected during the allergen season had statistically higher levels of markers for eosinophils, neutrophils, monocytes and CD8 lymphocytes compared to the off-season baseline. No change was observed for CD20 B-lymphocytes, total CD3 T cells and basophils. Subclassification of CD4⁺ T-helper cells demonstrated a parallel and significant expansion of Th2 and Th17 cells during the pollen season, while Th1 cells remained unchanged. Whereas absolute basophils numbers were unaltered, a significant increase of the basophil markers GATA2 and CPA3 was observed during the pollen season.

Conclusions: Apart from representing a positive method feasibility validation, our study provides further evidence of complex and parallel Th2 and Th17 immune signatures in seasonal AR. Our data also forward GATA2 and basophil CPA3 as potential biomarkers for ongoing allergic inflammation. It is thus proposed that the present histology-based approach, with its broad applicability, represents a powerful tool for decoding systemic immune alterations and guide novel biomarker strategies for improved personalized medication.

Key words: allergy, biomarker, peripheral leukocytes, allergic rhinitis, Th17, basophils

BACKGROUND

Allergic rhinitis (AR) represents one of the most common chronic medical conditions for seeking medical care(1). The disease is largely considered an IgE-mediated disorder with an allergic and Th2-mediated eosinophil-rich inflammation in the nasal mucosa as the main immunopathological feature(1-3). The typical symptoms are nasal itching /sneezing, rhinorrhoea, and nasal congestion. Globally, more than 400 million patients suffer AR and still today the disease is to a large extent underdiagnosed and undertreated. The pharmacological treatment for allergic rhinitis includes intranasal corticosteroids, oral and topical antihistamines and intranasal cromolyn(4-6). Additional treatment options are immunotherapy and, more recently, biologics targeting IgE (omalizumab)(7) or type 2 cytokines and their receptors (e.g mepolizumab, reslizumab, and benralizumab). Naturally, the use of costly biologics is predominantly for AR patients with concomitant type 2 and eosinophilic asthma and/or nasal polyposis. However, with more long-term safety data and the expected future price reduction, biologics will likely become a treatment option for a larger group of patients with AR. Indeed, improved treatment regimens are needed, because even with the best current pharmacotherapy up to 20% of the patients remain highly symptomatic.

One important factor to optimize treatment is the introduction of improved biomarkers. In particular, in the emerging era of personalized medicine, novel biomarkers are needed to inform about the immunological and inflammatory nature underlying the symptoms in individual patients. This approach is highly warranted since recent data has forwarded AR as a more immunologically complex disease than previously thought(4, 8). Thus, although the archetype allergic Th2 and IgE-driven eosinophilic inflammation plays an important role(2, 9), other separate and/or parallel immune responses may also contribute to the immunopathology. For example, it has recently been observed that neutrophil responses may be a significant feature of AR (10). Other reports provide evidence that also Th17 immune responses may contribute(11).

A range of biomarkers have been tested for use in aiding diagnosis, monitor disease activity and inform about inflammatory status in subjects with allergic rhinitis (8, 12-14). Plasma exudation, which is a hallmark of inflammation, can be analysed as extravasated plasma proteins like alpha-2 macroglobulin in nasal lavage fluids to yield a good umbrella parameter of ongoing inflammation (15, 16). Nasal lavage, or collection of more concentrated nasal surface secretions, have also been used to analyse individual immune cells or cytokines (17). However,

although informative, the use of such sampling is limited in clinical practice by its relative invasive nature and by technical challenges involved in normalising against variations in fluid recovery and by variations in nasal mucosal surface liquid viscosity. Assessment of routine blood samples overcome these challenges and has, despite only being a systemic measure, been used to monitor immunological markers and circulating leukocytes in AR (18-20). The assessment of peripheral blood leukocytes in AR has mainly been performed by flow cytometry (FACS). While this undoubtedly is a powerful and valuable method, tentative limitations include artefactual cell changes during the cell purification and that the cell marker to be analysed is limited and must be determined beforehand.

The present study explores a novel type of histology-based analysis of blood leukocytes as an alternative or complementary methodological approach for profiling circulating immune cells present in routine blood samples. Specifically, we test this approach on enriched blood leukocytes that have been subjected to minimal *ex vivo* handling before being fixed and embedded in paraffin for cutting-edge histological analysis. Comparisons are made between multiple leukocyte population markers in samples collected during and after the natural pollen season in patients with seasonal AR.

METHODS

Patients and Clinical Sampling

A total of 16 patients with seasonal AR were recruited to the study (mean age 25.6 years, range 20-39 years). Among the inclusion criteria was a history of seasonal AR for at least 2 years. Another requirement was a positive skin prick test to birch pollen allergen or other season-relevant allergen (15 out of the 16 patients were allergic to birch pollen and the total average wheal size was 5.9 mm, range 2-50). Exclusion criteria were chronic nasal disease and a history of asthma. Although most patients had more or less strict seasonal rhinitis, individuals with a positive skin prick test to house dust mite or animal dander were allowed to participate if they did not present any perennial symptoms and if they were not exposed to allergen-relevant animals. The study was approved by the local ethics committee (Swedish ethical review board, Lund: Dnr 2016/166) and informed consent was obtained from all patients.

Leukocyte profiles were explored in peripheral blood samples that were collected during the Swedish birch pollen season in the spring. Comparisons were made to control off-season

blood samples were obtained late in the autumn, several weeks after the last episode of the Swedish pollen season.

Assessment of Symptoms

Symptoms were recorded during the pollen season study period using validated diary cards(21, 22). The recorded symptoms, i.e. rhinorrhoea, nasal blockage and sneezing, were scored on a four-graded scale: grade 0, none; grade 1, mild; grade 2, moderate; and grade 3, severe symptoms. For each day, a total nasal symptom score (TNSS) was calculated.

Preparation of Blood Leukocyte Pellets

To make blood leukocyte pellets we slightly modified a protocol that previously has been shown to result in minimal *ex-vivo* handling artifacts or handling-evoked accidental cell activation(23)(Figure 1A). In brief, within minutes after the collection the 8 ml blood samples were immediately transferred into heparin tubes containing 16 ml of 4% buffered formalin (Histolab-REF:02176, 4%, pH7.4) and left for 1-hour at room temperature. To sediment red blood cells (RBC) from formalin-fixed whole blood, the blood sample was divided into two equal volumes of 12 ml and each portion was transferred into a tube containing 5 ml of dextran (5 % dextran + 3g glucose in PBS) and left for 1-hour at room temperature.

After 1-hour dextran-based RBC sedimentation, the leukocyte enriched plasma was carefully retrieved by a pipette and centrifuged at 1400 rpm (at room temperature 27°C) for 10 minutes. The obtained leukocyte pellet was resuspended into 4% formalin and transferred into a tube containing pre-warmed melted agarose (low melting agarose, Sigma-Aldrich A4018-10G) and again centrifuged at 1400 rpm for 10 minutes (at 27°C). The hardened agarose containing the leukocyte pellet was left in 4% formalin for approximately 12 hours (overnight) at room temperature before being subjected to standard dehydration (SAKURA-Tissue-Tek VIP® 6 AI automated dehydration machine) and embedding into paraffin pellets.

Immunohistochemistry

Paraffin-embedded blood pellets were sectioned at 4 µm thickness, and at least five blood pellet sections were placed per slide to create blood pellet micro array slides. As part of the antigen retrieval process, slides were baked at 60°C for 45 minutes and pretreated with pH-low (pH 6) in an antigen retrieval solution (Dako-DM829) in Dako PT Link machine (97°C for 20 minutes; PT-200, Dako/Agilent, Glostrup, Denmark).

A modified double and triple staining immunohistochemistry protocol was applied for simultaneous visualization of multiple immune cell markers(24, 25). Briefly, after antigen retrieval, endogenous peroxidase was blocked with peroxidase blocking reagent (Dako-DM821) for 10 minutes. Next, the slides were incubated with the primary antibody for 1 hour, washed extensively and thereafter incubated with an isotype-relevant secondary antibody coupled to a HRP-labeled polymer (Envision Flex-DM822 amplification kit) for 30 minutes before being subjected to a 10 minute incubation with HRP chromogen substrate. Excess remaining chromogen substrate and/or non-encapsulated HRP in the sections were denatured using 1:3 diluted denaturing solution (Biocare Medical- DNS 001L) for 5 minutes. After the denaturation step, a similar protocol round was repeated to achieve the double and triple staining. For double and triple staining, the following HRP chromogens were used; 3,3'-Diaminobenzidine (DAB-Dako-DM827, brown precipitate), vana green (Biocare-BRR807BC) and deep space black (Biocare- BRI4015 H, L). Finally, the slides were stained by hematoxylin (Htx) nucleus counterstaining and mounted in pertex (Pertex-Histolab). Information about the primary cell marker antibodies are presented in *Table 1*. Control experiments where the primary antibody was replaced with a non-relevant isotype control did not result in any staining. Importantly, for the comparison between off-season and season marker values, all sample preparations were identical and off-seasonal and seasonal samples were IHC stained and analyzed simultaneously.

Digitalization of Immunostained Leukocyte Pellets and Computerized Image Analysis

An automated Olympus VS-120 Virtual Slide Microscope was used for high resolution digitalization (20X lens magnification) of the entire section area from all multi-stained blood pellet sections. In each cell pellet the immunoreactivity for each stained cell marker (i.e. the detection chromogens), as well as the background Htx-stained cell nuclei, were automatically detected by color and spectral-based segmentation by computerized image analysis (Visiomorph software, Visopharm, Denmark) (*Figure 1 D*). Finally, for each cell marker, the total area (pixels) of the corresponding chromogen was calculated.

The areas of nuclei and immunostainings were combined to obtain the total area of blood pellet. Finally, the area of each particular marker was divided with the total cell pellet area and converted into a percentage of immunoreactivity.

In order to automatically detect double stained Th1, Th2, and Th17 CD4⁺ lymphocytes, each nucleus with positive staining for the relevant nuclear transcription factor (T-bet, GATA3,

and ROR- γ t, respectively) were detected by color segmentation. Next, any transcription factor-positive nucleus that was in direct spatial contact with surrounding CD4⁺ immunoreactivity was automatically reclassified as the corresponding Th subset (*Figure 1 E*).

Statistics

All the data were presented as scattergrams and mean values. As all the data were from the same sample population, The Wilcoxon test for paired observations was used for statistical analysis in Prism 8. The Pearson correlation coefficient analysis was performed to investigate correlations between cell markers, or between markers and symptom scores.

RESULTS

Method Optimization and Validation

Initial tests revealed that the current protocol of generating paraffin blood leukocyte pellets for histological analysis resulted in a well-preserved morphology of the cells as inspected after routine hematoxylin staining. Heat-induced antigen retrieval prior to IHC had a slightly negative effect on the morphology. Thus, initial studies, with different combinations of programmed time and temperature curves were tested in order to yield the combination that was best suited morphology preservation, robust IHC staining and computerized image analysis (*Figure 1B-C*). Our preliminary explorations also revealed that each cross sectioned pellet typically contained cross section planes of 5000 – 7000 individual leukocytes.

Using the optimized protocol with 97 °C HIER for 20 minutes, freshly cut sections from all season and off-season blood samples were simultaneously subjected to immunohistochemical staining and computerized image analysis. Importantly, despite that our type of quantification is conceptually different from conventional routine differential cell counts, the rough overall proportions of cell markers representing the major cell types was fairly similar to the expected overall leukocyte composition (*Figure 2B*). Further, of relevance to allergic diseases, routine clinical eosinophil differential counts correlated with the present histology-based enumeration of immunoreactivity for the eosinophil cationic protein marker EG2 (*Figure 2A*).

Altered Blood Cell Marker Immunoreactivity During the Allergen Season

Next, we performed a paired analysis comparing off-season (autumn) and allergic season (spring) immunoreactivity values for selected basic cell populations. Our data revealed statistically elevated levels of EG2 (eosinophils), CD68 (monocytes), and MPO (neutrophils) dur-

ing the spring compared to paired off-season autumn values (*Figure 2 C-I*). No statistical change could be observed for the basophil marker proMBP1, the pan T cell marker CD3, or the B-lymphocyte marker CD20 (*Figure 2*).

Increased Th2 and Th17 Blood Signatures

Assessment of T-lymphocyte subpopulation, including transcription factor-defined CD4⁺ T-helper subsets, demonstrated a statistically elevated value for CD8 immunoreactivity (i.e. cytotoxic T cells) compared to off-season values. Among the Th cell types CD4⁺, GATA3⁺ double positive Th2 cells and CD4⁺, ROR γ t⁺ Th17 cells increased during the pollen season, whereas no such change was observed for CD4⁺, Tbet⁺ Th1 cells (*Figure 3*).

Basophil-Related Markers

Basophils are classically linked to allergic disorders. Interestingly, although no statistical seasonal increase was observed for the basophil markers pMBP1 (*Figure 2F*), a robust statistical increase was observed for the basophil transcription factor GATA2 (*Figure 4 C*). A subset of the basophils has been described to also contain the granule proteases CPA3 and tryptase (26, 27). During the allergen season elevated blood leukocyte CPA3 but not tryptase could be detected together with a non-statistical trend towards increased surface bound IgE (*Figure 4A,D-E*).

Cell Marker Correlations

During the allergen season many cell markers seemed to be expressed independently within individual patients. However, a statistical correlation was present between EG2/eosinophils and the basophil marker GATA2 (*Figure 5A*). Intriguingly, a surprisingly strong correlation was also found between Th2 and Th17 lymphocytes (*Figure 5B*). Although our study only included a limited number of patients it allowed for some insight into the correlation between autumn and spring values among the various IHC markers. Some markers, such as blood leukocyte tryptase (i.e. basophil tryptase), EG2/eosinophils and the B cell marker CD20, was of similar levels within patients examined before and after the pollen season.

Despite that many of the cell markers were statistically increased during the spring pollen season, there was no clear correlations between leukocyte markers and the spring symptom scores.

DISCUSSION

The heterogeneous nature of the inflammation AR, and the emergence of new selective treatment options, call for improved ways to explore the immunological heterogeneity without invasive nasal sampling. This study presents such an approach and demonstrates that histological analysis of blood leukocyte pellets can be used for mapping circulating immune cell profiles. The fact that many immune cell parameters were statistically increased during the allergen season compared to off-season show the capacity of the present approach to monitor clinically relevant changes in inflammation status. Of particular interest was our finding of increased parallel Th2, Th17, neutrophil, and eosinophil responses during the allergen season. Our data also reveal a potential use of the basophil-associated markers GATA2 and CPA3 as markers of ongoing allergic inflammation. On a general note, the present approach for histology-based leukocyte mapping is certainly not restricted to allergic rhinitis and has bearings on most other inflammatory conditions.

From a methodological perspective, it is important to stress that the present approach to monitor blood immune cell marker is conceptually different from standard assessments of the leukocyte composition in peripheral blood. Thus, whereas FACS analysis and blood differential cell counts in routine clinical labs measure the proportions of whole cells, our approach rather measures the normalized area of actual staining (i.e. immunoreactivity). Naturally, the stained marker area per marker-positive cell area may differ between different types of markers. For example, the presently used nuclear transcription factors, cell membrane or cytoplasmic granule markers differ in respect to how much area of the entire cell profile that is actually stained. From this, together with marker differences in antibody dilution and staining intensity, it should be expected that the relative proportions between the present cell identification markers do not correspond to the exact proportions of cell numbers. Despite this, a surprisingly good correlation was achieved between the overall leukocyte proportions and between eosinophil numbers determined by a routine clinical lab and our parameter of EG2 immunoreactivity.

The present study demonstrates clearly that our methodological approach has the sensitivity to detect multiple allergen-evoked changes among circulating blood leukocytes. Among these, the significant eosinophil and Th2 responses were expected. Indeed, eosinophilia, which is a

hallmark of AR, and is thought to be driven by an underlying Th2 response. Of immunopathological relevance, allergen-induced immune responses not only stimulate eosinophil infiltration into the nasal mucosa but also their activation and cytotoxic mediator release through piecemeal degranulation (PMD) and programmed cytolysis (3, 21, 28). Perhaps seemingly more surprising was our observation of very robust increases in Th17 cells and MPO immunoreactivity, suggesting a significant IL-17 driven neutrophil response. This suggestion is further supported by the fact that IL17 cytokines like IL17A and IL17F stimulate immune cells and epithelial cells to release the neutrophil chemokines G-CSF and IL-8/CXCL8 (29, 30). Taken together, our findings thus agree with the several studies that suggest neutrophilia and mixed eosinophilic and neutrophilic phenotypes as a significant feature in AR(10, 19, 31). It is likely that the mechanism behind co-emergence of eosinophilia and neutrophilia in AR are multifactorial. Interestingly, among many alternatives, a recent report suggests that neutrophils in rhinitis patients may not only be down-stream effector cells but may also contribute to the allergic eosinophilia by priming T-cells to recruit eosinophils (10).

Basophils have been implicated in allergic diseases (26, 32) and basophil-associated biomarkers have an important role to e.g. predict response to immunotherapy (33-35). Basophil activation status in the blood is commonly analyzed by FACS as a translocation of CD63, CD69 and CD203c to the cell surface(36-38). Unfortunately, the present type of histological analysis cannot reliably differentiate between cytosol and membrane immunoreactivity. Hence, we explored five alternative basophil-associated markers; pMPB1, GATA2, CPA3, tryptase and surface bound IgE. While several of these may be expressed on other cells in inflamed tissues, they will be more or less unique to basophils in circulating blood(26). For example, most mast cells express GATA2, tryptase and surface bound IgE, and a significant proportion also expresses CPA3. However, mature mast cells are not present in the blood and mast cell progenitors are so exceedingly rare that they cannot have contributed to our blood analysis. The marker prp-MPB1 has been forwarded as a basophil pan marker but may also be present in eosinophil progenitors. Eosinophil progenitors, which are also expected to increase during the allergen season, also express GATA2 and may thus be regarded as a confounding cell in our assessment of basophils. However, when staining for CD34⁺ progenitor cells these were present in exceedingly low numbers (data not shown). Hence, neither eosinophils nor mast cell progenitors could have contributed significantly to our basophil marker analysis. Among these markers CPA3 and GATA2 values were significantly higher in-season than during off-season conditions. Although the biological background to this remain unknown, these

markers may therefore be added to the list of potential blood biomarkers for assessing allergic inflammation.

It should be noted that the main objective of the present study was to explore the potential and feasibility of the present histology-based approach for advanced blood leukocyte profiling of routine blood samples. We are aware that the number of the patients is limited and relatively small for this type of biomarker validation. We also noted that there was a rather marked variation among the included patients with regard to their symptom profiles. Nevertheless, the fact that our study design allows for paired analysis, and the fact that we did observe clear statistical season – off-season differences for several parameters, underscore the potential of this method. Indeed, one advantage over most other techniques is that the paraffin-embedded leukocyte pellets can be stored at room temperature for years and recut to make any type of follow-up analyses with a new round of immune markers. Although not explored here, advanced computerized image analysis may also be used to generate “FACS-like” data measuring staining intensity for all of the thousands individual cells typically present within a leukocyte pellet section. Naturally, the present methodology is fairly technically demanding and may not readily be suited for routine immune screening in a clinical setting. In spite of this, there is certainly an important role for more in depth immune cell profiling in the general goal to decode the immunological heterogeneity among AR patients. The type of data that is generated may also provide an important guidance to develop less complicated assays that can be tailored for implementation in real life clinical settings.

Conclusions

In summary, we present here a novel methodological approach allowing a robust and multifaceted mapping of circulating leukocyte population in routine blood samples. Apart from representing a positive feasibility validation of this approach, our study provides further evidence of complex and parallel Th2 and Th17 immune signatures in seasonal AR and a surprisingly robust upregulation of the blood basophil markers GATA2 and CPA3. Further studies applying these and other markers to larger patient cohorts is suggested to provide valuable insight into the immunological complexity of allergic rhinitis and other inflammatory conditions.

Table 1. Immune Cell Markers and Primary Antibodies

Antigen	Cell Type	Species	Dilution	Manufacturer and Clone
CD4	Pan T- helper lymphocytes	Mouse	1:50	Novacastra-NCL-L-CD4-368
T-Bet	Th1 cells (if CD4-positive)	Rabbit	1:1500	Santa Cruz -H210
GATA3	Th2 cells (if CD4-positive)	Mouse	1:1500	BD Pharmagen-L50-823
RORγt	Th17 cells (if CD4-positive)	Rabbit	1:50	Abcam-ab207082
MPO	Neutrophils	Rabbit	1:30000	Dako-A0398
CD20	B-lymphocytes	Mouse	1:1200	Dako-M0755
CD3	Pan T lymphocytes	Rabbit	1:120	Biocare
EG2	Eosinophils	Mouse	1:1000	Pharmacia. / EG2
GATA2	Basophils (tissue mast cells)	Rabbit	1:1200	Novus- NMP1-82581
CD68	Macrophages / monocytes	Mouse	1:50	Novacastra-NCL-L-CD163
IgE	Cells with surface bound IgE	Mouse	1:1000	DAKO
pMBP1	Basophils	Mouse	1:50	Biolegend
CD8	Cytotoxic T lymphocytes	Mouse	1:50	DAKO-C0/144B
CPA3	Basophils (+tissue MCs)	Rabbit	1:1000	Atlas-HPA008689
Tryptase	Basophils (+tissue MCs)	Mouse	1:1000	Millipore-MAB1222A

Legends

Figure 1. Schematic overview of the blood leukocyte pellet preparation (A). After blood sampling and a brief pre-fixation, cells are separated by dextran sedimentation (1). Layers containing leukocytes are collected and the white blood cells are spun down into pre-warmed melted agarose (2), which after being allowed to solidify and subjected to routine 4% formaldehyde fixation is embedded into paraffin (3) and cut into sections used for detection of multiple leukocyte markers by immunohistochemistry (exemplified in 4). Selection of optimal low pH heat-induced antigen retrieval conditions (B) and representative example of Htx-stained leukocytes amidst the few remaining erythrocytes (C). Example of detection and segmentation strategies for computerized quantitative image analysis (D-E). In panel E the brown ROR γ t – positive nuclei up to the left is touching CD4 immunoreactivity and is thus reclassified as a Th17 cell.

Figure 2. Correlation between clinical routine eosinophil cell count and computerized quantification of the eosinophil granule marker EG2 in blood leukocyte pellets (A). The overall composition and relative proportion of immunoreactivity for common leukocyte identity markers (B). Quantitative data of pairwise measurements of immunoreactive area in blood leukocyte pellets collected off-season/autumn and during the spring pollen season (C-I). Data shown are for major leukocyte population markers. * and *** denote a significance level of $p < 0.05$ and $p < 0.001$, respectively. Correlation in A is analyzed by Pearson correlation coefficient analysis.

Figure 3. Quantitative T-helper subset data of pairwise autumn and spring measurements for each individual patient (A,C,F). Th1, Th2, and Th17 populations were identified by double staining for CD4 and their corresponding transcription factor; i.e. T-bet, GATA3 and ROR γ t (B, D, E,G). * and ** denote a significance level of $p < 0.05$ and $p < 0.01$, respectively.

Figure 4. Pairwise leukocyte pellet data for the basophil-associated markers IgE, GATA2, CPA3 and Tryptase (A,C-E). ** and *** denote a significance level of $p < 0.01$ and $p < 0.001$, respectively. Representative example images of IgE-stained blood leukocytes (B). Triple immunofluorescence staining exemplifying a basophil, detected with the basophil marker BB1, and which is double positive for the granule proteases CPA3 and tryptase.

Figure 5. Scattergrams demonstrating the correlation between blood leukocyte pellet levels of eosinophils (EG2 immunoreactivity) and the basophil-associated transcription factor GATA2 (x axis in A). Correlations between blood pellet Th2 and Th17 cells during the pollen season are presented in panel B. The level of statistical correlation was tested with Pearson correlation coefficient analysis.

DECLARATIONS

Abbreviations

AR: Allergic rhinitis; TNSS: total nasal symptom score; HIER: heat-induced epitope retrieval; ISH: in situ hybridization; HRP: horse radish peroxidase; IHC: immunohistochemistry; PBS; phosphate buffered saline; CPA3: carboxypeptidase 3; CD: cluster of differentiation; Th: T helper; pMBP: pro major basic protein; MPO: myeloperoxidase; MC: mast cells; IgE: Immunoglobulin E

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Author's Contributions

LG, FN, and JE, conceived and designed the clinical study design: LG and FN coordinated the patient selection and clinical sampling. PS, MM and JE designed the histological studies. PJ, MM, PS, and JE validated the leukocyte preparation protocol. PS, GE, and MM performed the histological experiments and quantifications. JE and PS analysed and interpreted the data and wrote the manuscript. LG, FN, and LB contributed with data interpretation and manuscript editing. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The present study was approved by the local ethics committee (Swedish ethical review board, Lund: Dnr 2016/166) and informed consent was obtained from all patients.

Consent for Publication

All authors approved to the publication of the present study

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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B

Temperature	Time	Morphology	Antigenicity
70°C (A)	10 Mins	Good	Poor
80°C (B)	10 Mins	Good	Poor
97°C (C)	10 Mins	Good	Poor
97°C (D)	20 Mins	Good	Good

C

D

CD20 CD3 MPO

CD20 CD3 MPO

E

CD4 RORyt

CD4 CD4+RORyt CD4-RORyt

CD4 T-bet
GATA3

CD4 RORyt

Figures

Figure 1

Schematic overview of the blood leukocyte pellet preparation (A). After blood sampling and a brief pre-fixation, cells are separated by dextran sedimentation (1). Layers containing leukocytes are collected and the white blood cells are spun down into pre-warmed melted agarose (2), which after being allowed to solidify and subjected to routine 4% formaldehyde fixation is embedded into paraffin (3) and cut into sections used for detection of multiple leukocyte markers by immunohistochemistry (exemplified in 4). Selection of optimal low pH heat-induced antigen retrieval conditions (B) and representative example of Htxstained leukocytes amidst the few remaining erythrocytes (C). Example of detection and segmentation strategies for computerized quantitative image analysis (D-E). In panel E the brown RORyt – positive nuclei up to the left is touching CD4 immunoreactivity and is thus reclassified as a Th17 cell.

Figure 2

Correlation between clinical routine eosinophil cell count and computerized quantification of the eosinophil granule marker EG2 in blood leukocyte pellets (A). The overall composition and relative proportion of immunoreactivity for common leukocyte identity markers (B). Quantitative data of pairwise measurements of immunoreactive area in blood leukocyte pellets collected off-season/autumn and during the spring pollen season (C-I). Data shown are for major leukocyte population markers. * and ***

denote a significance level of $p < 0.05$ and $p < 0.001$, respectively. Correlation in A is analyzed by Pearson correlation coefficient analysis.

Figure 3

Quantitative T-helper subset data of pairwise autumn and spring measurements for each individual patient (A,C,F). Th1, Th2, and Th17 populations were identified by double staining for CD4 and their

corresponding transcription factor; i.e. T-bet, GATA3 and RORyt (B, D, E,G). * and ** denote a significance level of $p < 0.05$ and $p < 0.01$, respectively.

Figure 4

Pairwise leukocyte pellet data for the basophil-associated markers IgE, GATA2, CPA3 and Tryptase (A,C-E). ** and *** denote a significance level of $p < 0.01$ and $p < 0.001$, respectively. Representative example images of IgE-stained blood leukocytes (B). Triple immunofluorescence staining exemplifying a basophil,

detected with the basophil marker BB1, and which is double positive for the granule proteases CPA3 and tryptase.

Figure 5

Scattergrams demonstrating the correlation between blood leukocyte pellet levels of eosinophils (EG2 immunoreactivity) and the basophil-associated transcription factor GATA2 (x axis in A). Correlations between blood pellet Th2 and Th17 cells during the pollen season are presented in panel B. The level of statistical correlation was tested with Pearson correlation coefficient analysis.