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Innovative robust basophil activation test using a novel gating strategy reliably diagnosing allergy with full automation

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Short title:

Robust high-throughput BAT to reliably diagnose allergy

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JB, CS, TS, SK and UJ designed the study. JB, CS, MH and SK performed experiments and acquired data. JB, CS, MH, SK and TS analyzed data. JB, CH, TS and UJ wrote the manuscript. UJ obtained the positive votes from the ethics committee, recruited and characterized the patients and control individuals and raised the third party funding.

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None of the authors had any financial relationships for themselves and their immediate family/significant others.

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Abbreviations:

APC, Allophycocyanin; BAT, basophil activation test; CCR3, C-C chemokine receptor type 3; CD, cluster of differentiation; CV, coefficient of variation; Cy7, Cyanine7; EAACI, European Academy of Allergy and Clinical Immunology; Em_{max}, emission maximum; FMLP, formyl-methionyl-leucyl-phenylalanine; FSC, forward scatter; IL, interleukin; MFI, median fluorescence intensity; MQ10, MiltenyiBiotecMACSQuant 10; PBS, phosphate buffered saline; PE, R-phycoerythrin; pps, percentage points; ROC, receiver operating characteristic; RT, room temperature; SOP, standardized operating procedure; SSC, side scatter; TS, time series;

ABSTRACT

Background: Allergy is one of the most common chronic diseases in Europe. Therefore, an increased need for specific and sensitive diagnostic tests that truly detect allergy exists. This study aimed at establishing a highly specific high-throughput and automated basophil activation test (BAT) that proves the existence of an allergy with utmost probability.

Methods: BAT from 1,104 samples were analyzed, a novel gating strategy with three antibodies (FcεRIα, CD203c, CD63) was established and compared with our published protocol (12 antibodies). Based on the novel gating strategy, storage conditions, automated measurement, and analyzes using R (1,376 samples out of 1,389) were optimized to set up a high-throughput BAT.

Results: No differences in sensitivity and specificity were found between the novel three antibody (FcεRIα, CD203c, CD63) and the 12 antibody gating strategy or between automated and manually analyzed samples (saving up to 90% of labor time). The time frame for basophil activation measurement after blood donation has been extended considerably (whole blood storage ≤ 7 days (RT) and 17 days (4°C) prior to BAT preparation and measurement). Respective storage conditions were optimized for samples after stimulation, staining and preparation (≤ 7 days (RT) and 28 days (4°C)). These achievements were confirmed by a nationwide ring trial showing robustness and applicability of our BAT on a variety of flow cytometers.

Conclusions: Our considerable optimizations overcame the hurdles that until now prevented the BAT from being used as high-throughput allergy diagnostic test in routine laboratories, and shall allow for collaborative studies between clinics and research centers.

Keywords: allergy diagnostic, clinical trials, oleosins, peanut allergy, sensitization

1. INTRODUCTION

Allergic disorders are one of the most common chronic diseases in Europe, and many patients are not adequately treated due to misdiagnosis.¹ Skin prick test (SPT) and immunoglobulin (Ig)E antibody assays detect only sensitization but do not prove allergy. In order to prove allergy in unclear cases, challenge test with the allergen source is currently diagnostic gold standard.

However, the basophil activation test (BAT), which mimics the allergic reaction *in vitro*, has manifold advantages as diagnostic test: good safety profile, high sensitivity/specificity, and the potential to predict the severity of an allergic reaction.²⁻⁴ Importantly, it allows discrimination between sensitized asymptomatic and truly allergic individuals.⁴⁻⁸ Thus, the BAT has the potential to replace expensive and risky allergen challenge tests.^{7,8} Yet, no consensus has been found as to which of the many BAT protocols should be applied to obtain comparable results through their harmonization.⁹⁻¹⁴ Minimalistic selection of identification markers, for example, causes high cut-offs, due to contaminations with other cells.^{11,15-17} So far, only the use of two activation markers (CD63, CD203c), is widely accepted.¹⁸⁻²⁰

In our previously published BAT protocol using 12 antibodies, high sensitivity and specificity (100%), discriminating between allergic and sensitized but asymptomatic individuals, with a low cut-off for positivity were achieved.⁷ This protocol, too expensive and complex for routine diagnostic settings, forced us to simplify and expand our BAT protocol without affecting its excellent diagnostic performance. Together with an automation of sample preparation, measurement, data analysis, and elongation of the time span between blood donation and sample processing/measurement we enabled the feasibility of our protocol in a nationwide ring trial.

2 MATERIAL AND METHODS

2.1 Study approval

The study was approved by the local ethics committee of the University of Lübeck, Germany (approval nos. 10-126, 16-268, and 13-136). All study participants gave written informed consent.

2.1.1 Study populations

Patients with a convincing history of peanut allergy and controls (A, sensitized but asymptomatic as well as B, non-allergic individuals), were continuously recruited between 2015 and 2020 in Borstel and Lübeck (Germany). After a thorough clinical history supported by a standardized questionnaire, the status of the study population was confirmed by SPT, serology and/or basophil activation test (Tables S1-3). Heparinized whole blood from the study population was used to perform the basophil activation test (BAT). Detailed demographic characteristics of study subjects included in BAT experiments are listed in Tables S1-3.

2.2 Allergens and stimulants

The following allergens were either isolated and purified from natural (n) sources or recombinantly expressed (r) in *E. coli* BL21(DE3) and purified at the Research Center Borstel (Borstel, Germany): peanut oleosins (n) and defensins (n), Der p 2 (r/n), Bet v 1 (n), Ara h 8 (r), Ara h 14 (r), Ara h 15 (r). The identity of each allergen was verified on a QExactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, Waltham, MA). Further allergens were purchased from Indoor Biotechnology Ltd. (Cardiff, UK): Ara h 1 (n), Ara h 2 (n), Ara h 6 (n). Peanut extract was prepared according to the protocol of Boldt et al.²¹ The stimulants formyl-methionyl-leucyl phenylalanine (FMLP 1 µM, Sigma-Aldrich, Steinheim, Germany), anti-IgE (1:1 mixture of goat anti-human IgE (1 µg/mL, Sigma-Aldrich, Steinheim, Germany), and goat anti-human IgE (1 µg/mL, Abcam, Cambridge, UK) were used as positive controls for the BAT, PBS as negative control.

2.3 Flow cytometric instruments and antibodies

Measurements were conducted on an LSRII instrument (BD Biosciences, San Jose, California, USA), of which 3 lasers and 7 (12 antibody gating strategy) or 3 (three antibody gating strategy) detection bandpass (BP) filters were used for manual data acquisition (configurations listed in Table S4).

Automatic sample preparation and measurement were performed on a MACSQuant10 (MiltenyiBiotec, BergischGladbach, Germany). For the inter-laboratory testing 10 further flow cytometers (BD Biosciences) were involved: 3x FACSCantoII, 2x LSRII, 2x Fortessa, 1x Symphony, 1x FACSLyric and 1x FACSCalibur (configurations listed in Table S4). A detailed description of used antibodies for different protocols (3 antibody protocol, 12 antibody protocol, and CCR3 protocol) is documented in Table S5.

2.4 Basophil activation test (BAT)

The 12 antibody BAT and the identification of basophils (gating) was performed as previously reported.⁷ Although interleukin (IL)-3 is often used as priming agent, we refrained from using it. For the evaluation of the three antibody gating strategy (three antibody BAT), the acquired data of the 12 antibody BAT were taken and cells gated as shown in Figure S1A. Analysis was conducted with the same fixed quadrant for the CD203c vs. CD63 gating for the 12 and the three antibody evaluation (Figure S1B). The work-up of the BAT with CCR3 was the same as for the 12 antibody protocol with the exception that a reduced set of 4 anti-human antibodies (Biolegend, Fell, Germany) was used (CCR3-BV421, CD45-BV510, CD203c-PE and CD63-APC). Basophils were identified as shown in Figure 1A and their activation status analyzed. The total numbers of detected basophils were recorded.

2.5 Automation process

For the automation process, a sufficient amount of lysis buffer was prepared first, and the tubing of the storage solution was moved into the lysis buffer reservoir. After that, the flow cytometer was flushed extensively with lysis buffer. Furthermore, the anti-human antibody mixture was prepared

(three antibody mixture: FcεRIα-PE/Cy7, CD203c-PE and CD63-APC) in a dark vial which was placed on the reagent rack 4 of the MACSQuant. Stimulants (15 µl) were then placed in a 96-well deep well plate (Sarstedt AG & Co. KG, Nümbrecht, Germany) and mixed manually with 135 µl of fresh heparinized blood. Thereafter, the plate was mounted onto the 96-well rack (chill 96) of the MACSQuant. Next, the run was started with the following parameters: flowrate (high), mix sample (medium), mode (fast), uptake volume (450 µl), and sample volume (150 µl). After 30 min of incubation time with the stimulants, 50 µl of antibodies were automatically added. 20 min later, 400 µl of lysis buffer was added by the instrument, and the samples were incubated for 25 min. Thereafter, 600 µl of MACS running buffer was added, and the samples were measured by the instrument.

2.6 Time series measurement (TS1): one time sample work up and continuous analysis

Heparinized whole blood from 16 study patients (10 allergic patients, 3 sensitized but non-allergic subjects and 3 non-allergic individuals) was stimulated (PBS, anti-IgE, or allergen, 5,000 ng/ml) and prepared for measurement according to our published standard protocol (12 antibody BAT).⁷ The samples were measured on the day of preparation and the results designated as day 0 reference. Thereafter, samples were split, and one half of the samples were kept at room temperature (RT, 19°C) and the other half at 4°C, both under the exclusion of light. All samples were measured each day in week 1 and then twice a week for 3 more weeks on our LSR II.

2.7 Time series measurement (TS2): blood donation and repeated work-up

Heparinized whole blood from 5 study patients (3 allergic patients, 1 sensitized but non-allergic subject and 1 non-allergic individual) was sampled, partially stimulated (PBS or allergen, 5,000 ng/ml), and prepared for measurement according to our published standard protocol (12 antibody BAT) multiple times.⁷ The initially donated blood samples were split into two parts, which were stored at 4°C and RT, respectively. Starting at one day after the blood donation, a portion of each remaining blood sample was stimulated, prepared and measured daily, every day in week 1 and then twice a week for 2 more weeks on an LSR II. As for the previous experiment (TS1), we had to

discontinue the measurements of blood that was stored at RT after day 7, because of the fast decline of viable basophils that could respond to the allergen stimulation.

2.8 Statistics

Medians, means and standard deviations were calculated using MS Excel (Microsoft, Redmont, USA). Data analysis and statistics were conducted using GraphPad Prism software package (version 6, GraphPad Software, San Diego, CA). Comparison between basophil markers (Figure 1C) and flow cytometric instruments (Figure 5) was performed using the Mann-Whitney *U* test. Calculation of the optimal CD63-cut-off value was done using ROC (receiver operating characteristic) analysis (Figure 6C). Comparison of the activation levels and the total number of basophils (Figure 6A, B), was performed using the Wilcoxon signed-rank test.

2.9 Further Methods

Inter-laboratory survey, and *Evaluation of the BAT using an R script* are described in detail in the Supplementary Appendix (Supplementary Methods).

3 RESULTS

3.1 Choice of basophil identification markers

At first, we focused on specific basophil markers (CD203c, FcεRIα) from our original protocol.⁷ Although CD203c is used manifold as activation marker to distinguish allergic from healthy individuals, this marker is also constitutively expressed on basophils.²²⁻²⁴ Therefore, we used CD203c in our set up as marker for identification and not as a marker for activation. Further we were looking for markers, which expression intensities remained relatively constant to achieve a robust and clear distinction between basophils and other cells. We tested CD203c, FcεRIα and CCR3 (C-C chemokine receptor type 3), the latter marker already used in commercially available BAT kits and used here as a benchmark.

When following the gating strategy CCR3 vs. side scatter (SSC) used by many investigators, a discrete basophil population appeared for PBS (phosphate buffered saline, Figure 1A) to set up a proper basophil gate.^{8,14,25-28} However, a markedly reduced CCR3 expression was observed upon stimulation of basophils (Figure 1A; anti-IgE #1). This effect becomes even more apparent when looking at the CCR3 expression of PBS, anti-IgE and FMLP (formyl-methionyl-leucyl-phenylalanine, Figure 1B). Here, a perceptible shift to lower fluorescence intensities can be observed for anti-IgE and FMLP.

To compare the overall robustness of different basophil markers, we investigated the relative median fluorescence intensity (MFI) of samples stimulated with anti-IgE or FMLP and PBS (Figure 1C). Differences were only observed in samples using CCR3. This stimulation-associated downregulation of CCR3 becomes particularly problematic in cases of an insufficient erythrocyte lysis: erythrocytes hamper gating of a discrete basophil population (Figure 1A, anti-IgE #2).

Therefore, we fundamentally revised previously established protocols and developed a new gating strategy using CD203c in combination with FcεRIα as robust basis to identify basophils (Figure S1A).

3.2 Development of a robust two identification marker-based gating strategy

Two markers for basophil identification together with CD63 (activation) bound to bright fluorochromes (CD203c-PE, FcεRIα-PE-Cy7, and CD63-APC) were chosen and a novel gating

strategy established (Figure S1), which resulted in properly gated basophils (Figure S1A, IV).²⁹ They were analyzed for contamination with other cell types (Figure 2A), which was very low (0.1% (PBS), 0.2% (anti-IgE), and 0.5% (FMLP), respectively) when compared with the application of the current CCR3 protocol. CCR3^{high}/SSC^{low} basophil populations showed contamination with cells, which did not express the basophil marker CD203c (Figure S2). The percentage of non-basophil cells was 20% (PBS), 33% (anti-IgE), and 28% (FMLP), respectively (Figure 2B).

To evaluate the reliability of our new protocol, we compared it to our high performance 12 antibody protocol (Figure S1B, and cited literature).⁷ For this purpose, 1,104 samples were analyzed by both protocols with regard to the number of basophils and their activation. No significant difference was found for the total number of basophils (Figure 2C, allergic patients), which median was 0.5% (5% quantile: -10.3 percentage points (pps) and 95% quantile: 9.9 pps). Comparing basophil activation, we found no significant differences between the results of both strategies, indicated by high correlation coefficient ($R^2 = 0.997$, Figure 2D) and a median of 0 (5% quantile: -1.56 pps and 95% quantile: 1.82 pps). The three antibody protocol showed the same high diagnostic sensitivity and specificity as the 12 antibody protocol, but saved 86% of expenses (as calculated using list prices).

3.3 Elongated time frame for measurements

BAT samples should be analyzed <24 hours after blood donation, but first data indicate that short-term storage of blood did not compromise subsequent basophil activation.^{27,30-33} We analyzed prepared samples (time series (TS)1) or whole blood (TS2) after different storage periods.

To analyze the reactivity of basophils in prepared samples over time (TS1), we took blood samples from 16 individuals (10 patients allergic to either birch pollen, house dust mite, or peanut, respectively; 3 sensitized but non-allergic individuals and 3 non-allergic subjects), stimulated them (PBS, allergen and anti-IgE) once at day 0 and analyzed these samples over a period of 4 weeks, for at least twice a week (see Figure 3A and Figure S3). In order to find out whether storage conditions have an influence on the BAT results, we split the initially prepared samples and stored one part at 4°C and the other at constant room temperature (RT, 19°C) under exclusion of light to prevent fluorochrome degradation.

TS1: Activation of basophils changed slightly (4°C, Figure 3A) over the course of 28 days, whereas it changed after 7 days in samples stored at RT (Figure S3). The median increase of activation (allergic individuals, 4°C) by allergen was below 5 pps over a period of 28 days. A continuous rise of activation over the first days to 32 pps at day 14 was seen (RT). Non-allergic subjects and only sensitized individuals showed a slight increase (mean 1.4 pps after 28 days) in activation (4°C) and an increase starting at day four after allergen-stimulation (RT, mean 6.3 pps after 14 days). Background signal (PBS control) was unaltered (4°C) or increased slightly over time (RT). Storage at RT was accompanied by a continuous dying of the basophil population that forced us to discontinue the measurements of the samples stored at RT after day 14.

After having obtained these promising results, we also wanted to know whether donated blood could be stored for a prolonged period of time without affecting the classification of the tested individuals (TS2). Therefore, we sampled blood from 5 individuals (3 patients allergic to house dust mite, 1 non-allergic subject and 1 sensitized but non-allergic individual), prepared (stimulation and staining) and analyzed the stored blood multiple times over a period of 3 weeks. To study the impact of the storage temperature, the initially donated blood samples were split into two parts, which were stored analogously to the prior experiment (at 4°C and RT, respectively).

TS2: Whereas activation varied over the investigated period (patient #1 (Figure 3B) and #2 (Figure S4)), it steadily declined for patient #3 (Figure S4). However, diagnosis of allergic patients was possible within 17 days (blood storage at 4°C). No difference in the activation levels of the basophils were observed for the control individuals. As for the previous experiment (TS1), we had to discontinue the measurements of blood that was stored at RT after day 7, because of the fast decline of viable basophils that could respond to the allergen stimulation.

To verify our observations, we initiated a field experiment in which the basophil activation in samples from 3 allergic blood donors was investigated prior and after postal shipment (Figure 3C). Fresh donor blood was split into 6 parts, whereas one part was prepared (stimulation and staining) and analyzed on the same day and the other 5 parts were handed over to 5 different local post offices. After submission of the samples to the local distribution center, samples were delivered back to our laboratory, prepared (stimulation and staining) and analyzed as quintuple determinations 3 days after initial blood donation. We observed slight disparities in activation levels, but the background signal

(PBS) was low, and activation induced by allergen was always distinguishable from background. Therefore, allergic study participants could be correctly identified, despite the fact that the blood has not been processed prior to the shipment via different post offices. Therefore, the results of our BAT protocol demonstrate the feasibility of whole blood sample shipment to other laboratories allowing them to perform the BAT with reproducible results.

3.4 Inter-laboratory testing (ring trial)

We wanted to assess the robustness of our new protocol in cooperation with 9 other laboratories (six different flow cytometers, Table S4). For this purpose, we divided prepared samples (PBS, anti-IgE or allergen) and sent them via conventional mail to different core facilities within Germany (Figure 4A). A detailed protocol (Supplementary Appendix, Protocol S1) with calibration beads to set up different instruments, was provided together with the samples. Overall, the desired number of basophils (800) was recorded at all instruments, and activation of basophils was detectable. With regard to the results (Figure 4B), minor differences for basophil activation were observed, comparing our data with those from our cooperation partners. The mean difference for samples incubated with PBS, anti-IgE and allergen were 0.43 pps (range 0.01 to 2.33 pps), 0.63 pps (range 0.01 to 5.28 pps) and 1.60 pps (range 0.05 to 11.07 pps), respectively. Moreover, we generally observed a very low background for all investigated individuals (mean 0.28%).

3.5 Automatic sample processing and measurement to reduce hands-on time

Integration of the BAT into routine diagnostics is largely hampered by enormous laboratory work as all commercially available BAT kits need to be performed in tubes, which impedes a high-throughput measurement of samples. Therefore, we established an automatic sample processing and measurement using a flow cytometer with integrated robotic functions (MACSQuant10 (MQ10), MiltenyiBiotec GmbH, BergischGladbach, Germany). After having manually performed incubation of blood and stimulants in a 96-deepwell plate, the plate was transferred to the 96-well plate holder on the instrument. Here, the automated protocol (Supplementary Methods) was conducted by the MQ10. To assess the feasibility of this approach, we compared our published manual sample preparation to

automated work-up using the same blood samples in parallel (Figure 5).⁷ Although measurements were conducted on two different flow cytometers (manual: LSRII; automated: MQ10, according to our standardized operating procedure (SOP)), there was no difference using the manual or automated sample preparation. However, using the automated protocol reduced average hands-on time for 96 samples from about 9 hours to 1.5 hours, which saved more than 80% of manual work-up.

3.6 Development of a robust automated analysis template using R

Based on 1,389 individual BAT samples, we created a data-driven automatic analysis algorithm using Bioconductor tools in R (Supplementary Methods). Although the automatic approach was able to identify even small numbers of basophils correctly, we implemented a threshold of 100 basophils as minimum requirement for analysis by the algorithm (Figure S5). Thus, 1,376 out of 1,389 samples (99.1%) could be analyzed automatically. The comparison of automatic assessment to manual evaluation by an expert showed a very good agreement with respect to the activation level (Figure 6A) and total number of basophils (Figure 6B), resulting in a correlation coefficient of 0.952 and 0.967 ($P < 0.001$), respectively. Automatic analysis resulted in the same diagnostic sensitivity (100%), but a slightly lower specificity (97%) for peanut-allergic patients (Figure 6C) compared to our previous results.⁷ Using our automated assessment all patients at risk are identified correctly, and only one out of 30 non-allergic controls will be advised to avoid peanuts due to an exaggeration of activated proportion of basophils by the algorithm (1.04 pps above the cut-off, Figure 6C).³⁴ However, results of the algorithm were almost identical to that of manual gating using the 12 or three antibody protocol conducted by an expert (Figure 6D). Additionally, for automatic analysis, we implemented a visual quality control and quality checks to identify technical errors/problems (Figure S5, and Supplementary Methods) derived from the sample preparations.

4 DISCUSSION

The dramatic increase of allergic diseases has led to a growing need for reliable high-throughput diagnostic tests that do not simply detect the presence of allergen-specific IgE but also reveal its biological consequences.^{2,6,8,17,35} The basophil activation test (BAT) has an enormous diagnostic potential, but its application in routine diagnostics is largely hampered by many hurdles (e.g. robustness), which we addressed here. Many BAT protocols claim to be sufficient with only one single surface marker exploited.^{9,10,12,14,22,26} For example, CCR3 can be sufficient for non-stimulated samples (Figure 1A), but due to stimulation-induced decrease of CCR3 expression, the basophil population is contaminated with non-basophil cells with a subsequent underestimation of their activation level. Therefore, the value of CCR3 is still a matter of debate.³⁶⁻³⁹

We addressed this problem. Our novel protocol together with our novel gating strategy has been shown to be a robust approach that allows identification of pure basophil populations (CD203c and FcεRIα) and the evaluation of their activation (CD63 expression). Our gating strategy includes doublet exclusion that is not yet used by any of commercially available BAT kits. Using bright fluorochromes (PE, PE-Cy7, APC) with an optimized low spillover further advances analysis of basophils (Figure S6).²⁹ Most flow cytometers can be addressed with our protocol as they operate with the required lasers (488nm, 633nm). There are minor spillover effects caused by the combination of PE/PE-Cy7, but these neither influence the analysis nor the outcome of measurements. In fact, there was no need for compensation, which normally has to be done carefully.^{14,30}

To prove diagnostic reliability of our new protocol, a data set of more than 1,104 samples (51 study patients) was reanalyzed with regard to number and activation level of basophils and then compared to the results of our highly specific and sensitive 12 antibody protocol.⁷ The obtained data were almost identical to those already published, resulting in similar ROC-curves and demonstrating high diagnostic sensitivity (100%) and specificity (97%) of our test (Figure 6C and cited literature).⁷ This further demonstrates that our protocol dramatically reduces complexity and costs (86% saving) of BAT without affecting its superior diagnostic performance. Moreover, the results were achieved without using any extrinsic stimulants such as interleukin (IL)-3, which have been denoted as expendable by the European Academy of Allergy and Clinical Immunology (EAACI).^{32,40,41}

A major hurdle for implementation of the BAT into routine diagnostics is the requirement to analyze blood samples within a few hours after donation a limitation that has already been addressed by other BAT-specialists. Their results showed that a delayed analysis of samples (after stimulation, staining and preparation) even after 5 days of storage may be feasible if using CD63 expression as a marker for basophil activation.^{30,33}

To examine the limits of prepared samples and the storability of blood, respectively, we initiated two independent time series (TS1, TS2). Our results (TS1) show that measurement of stored samples after stimulation, staining and preparation is possible for several days (RT) and up to at least 28 days (4°C). Furthermore, we have shown that there is no need to analyze whole blood within 24h (TS2). A correct classification of study participants was still possible after 7 days (RT), or after 17 days (4°C). A small real-world pilot study (shipping blood samples prior to BAT preparation and measurement) revealed comparable tendencies as in the TS2, namely only a small alteration in basophil activation level. This issue might be, in parts, attributed to daily preparation of stimulants and buffers, which underlie unavoidable variations. Our observations confirm findings of others and stress the fact that the currently propagated limited time span of <24h until sample measurement (stored samples after preparation or whole blood) needed to be re-evaluated.^{30-32,42}

A nationwide ring trial evaluated the robustness of our protocol and verified practical usefulness of our findings on the prepared samples. Overall, an inter-assay coefficient of variation (CV) of 7.82% for IgE and 6.71% for allergens was calculated. It is of note that the samples were transported without cooling and had been measured between three and seven days after blood donation and preparation. This outcome provides substantial data that transfer of samples to external institutions is possible without a negative influence on data quality, so that our protocol provides a beneficial approach for scientists and clinicians without direct access to flow cytometers, opening up new dimensions of cooperative studies, clinical trials, and routine measurements in analytical laboratories.

BATs routine application in medical care units and analytical laboratories is further hampered by time-consuming and laborious handling of samples in single tubes (using commercial BAT kits). Therefore, we transferred our novel protocol to a flow cytometer with robotic functions. Only preparation of stimuli and addition of blood have to be done manually. All other steps are fully

automated, which is, to best of our knowledge, the first approach to realize a high-throughput BAT. In direct comparison with our manual protocol, we observed no differences in activation levels of basophils (Figure 5), but saved over 80% of labor time.

Besides automated sample processing, we aimed to automatically analyze acquired BAT data. This is the second attempt to implement a data-driven algorithm for BAT – the first is based on a CCR3 protocol, which has been shown to be less robust compared to our new gating protocol.⁴³ Poor basophil identification by the algorithm might be one reason for the almost 10 times higher number of experiments requiring manual gating compared to our results (8.5% vs. 0.9%), which are obtained by robust basophil identification markers. Several conditions changed during the study period of our three antibody protocol (e.g. photomultiplier tubes, staff members), but the results of the data-driven programmatic analysis are comparable with the results obtained by manual gating of an expert (Figure 6). This automated data analysis template will reduce analysis time of approximately one hour down to the transfer of acquired data to analysis server. Ideally, such an algorithm could be integrated in the instruments' software in future to provide an all in one solution for operators.

As with every study, there are some limitations to the research presented here. First, the number of investigated allergic participants and controls is relatively small, but the ones included are well-characterized. Second, experiments were mainly performed on the same flow cytometer at our facility. However, the data obtained from our ring trial indicate that variations between flow cytometers can be minimized by thorough calibration of the instrument. Investigations including more and other single allergens in BAT with samples from patients with different allergy severity grades (class I and class II food allergy) are already underway.

In conclusion, based on a robust novel gating strategy we have developed an automated high-throughput highly discriminative basophil activation test protocol that drastically reduces hands-on time as well as analysis time without a flow cytometric expert, opening up possibilities for multicenter studies and the routine use of BAT as allergy diagnostic test.

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FIGURE 1. *Analysis of stimulation-induced shift of basophil markers.* **A**, Identification of basophils by applying the CCR3 gating strategy (CCR3^{high}/SSC^{low}) used upon PBS samples. Two selected anti-IgE stimulated samples (anti-IgE #1 and anti-IgE #2) show difficulties to adjust the recommended gating strategy (especially anti-IgE #2: distinction between basophils and other blood cells is impossible). **B**, histograms of CCR3 expression (relative fluorescence intensities): unstimulated PBS samples and those stimulated with anti-IgE and FMLP. **C**, changes of the median fluorescence intensities (MFIs) upon stimulation with anti-IgE or FMLP for the basophil markers CCR3, CD203c and FcεRIα. Study subjects included in the respective experiments are listed in Table S1. Red bars indicate medians of the group. Statistical significances were determined using the Mann-Whitney *U* test: **P*<0.05.

FIGURE 2. *Comparison of basophil gating strategies.* **A**, analysis of the identified basophil population after application of our two identification marker-based gating strategy (using our three antibody protocol, Figure S2) for contaminations with other cells using anti-lineage cocktail antibodies (anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, anti-CD56) and anti-HLA-DR antibodies in samples stimulated with PBS, FMLP and anti-IgE. Cells in the red box of the inserts (representative gating) are considered as impurities. **B**, analysis of the identified basophil population (CCR3^{high}/SSC^{low}, gating Figure 1A) using the CCR3 protocol by gating for the basophil identification marker CD203c (Figure S2). The number of CD203c⁺ is presented, indicating contaminations with non-basophil cells. **C**, a comparison of the number of identified basophils after application of our 12 and three antibody protocol, respectively (allergic patient no. 1-21). Means and standard deviations are indicated. **D**, comparison of the basophil activation level (% CD63 expression of identified basophils; PBS, anti-IgE and FMLP samples) between the 12 and the three antibody protocol assessing 1,104 individual samples (51 individuals: 21 peanut-allergic patients, 15 peanut-sensitized subjects without clinical symptoms, and 15 non-allergic (healthy) individuals). Study subjects included in the respective experiments are listed in Table S1. Red bars indicate medians of the group.

FIGURE 3. *Evaluation of BAT after storage of prepared samples and whole blood.* **A**, TS1: progression of the basophil activation (% CD63 expression of identified basophils) for prepared

samples stored at 4°C within a time period of 28 days (10 patients allergic to either birch pollen, house dust mite, or peanut (each sample being stimulated with an allergen concentration of 5,000 ng/ml), respectively; three sensitized but non-allergic individuals and three non-allergic subjects; PBS: circle, anti-IgE: triangle, allergen: square). Data show changes (in percentage) of activation in comparison to direct measurement at day 0. **B**, TS2: progression of the basophil activation (% CD63⁺) for whole blood samples (PBS: circle, Der p2 (in a concentration of 5,000 ng/ml): square) from a house dust mite-allergic and a sensitized but non-allergic individual over a period of 21 days. Blood was stored at 4°C (blue) or RT (red), then stimulated (PBS: circle, allergen: square) and prepared for analysis at each of the given days. **C**, fresh donor blood was split into 6 parts. Whereas one part was analyzed on the same day, the other 5 parts were handed over to 5 different local post offices. BAT results (% CD63⁺) of whole blood samples from three allergic donors (Bet v 1, Der p 2, peanut oleosins: each in a concentration of 5,000 ng/ml at the day of blood donation and preparation (blue, quadruple determination) as well as after postal delivery and preparation (red, quintuple determination)). Study subjects included in the respective experiments are listed in Tables S1, S2, and S3. Red bars indicate medians of the group.

FIGURE 4. *Results of the ring trial evaluation following the postal delivery of prepared samples.* **A**, a map of Germany showing the locations of the cooperation partners and specification on the respective instruments involved in the ring trial. The longest route for postal delivery (Borstel (■) to Constance (●)) was about 900 km. **B**, the basophil activation levels (% CD63 expression of identified basophils) of prepared samples (PBS, anti-IgE or allergen (1x Bet v 1 [major allergen in birch], 3x Der p 2 [major allergen in the house dust mite *Dermatophagoides pteronyssinus*], 1x peanut oleosins; each in a concentration of 5,000 ng/ml)) from 5 blood donors measured on 11 flow cytometers at 10 different laboratories (Borstel: ■, external sites: ○ (each color corresponds to the location visualized within **A**)). Study subjects included in the respective experiments are listed in Tables S1, S2, and S3.

FIGURE 5. *Comparison of automated and manual sample preparation and measurement.* Blood from 3 allergic donors (1x Bet v 1, 1x Der p 2, 1x peanut oleosins; each used in a concentration of 5,000 ng/ml) was in parallel subjected to manual and automated preparation and the basophil activation (% CD63 expression of identified basophils) analyzed thereafter. Automated analysis was performed on a

MQ10 and measurement of manually prepared samples on an LSRII. Study subjects included in the respective experiments are listed in Tables S1, S2, and S3. Red bar indicates the median for each group. There was no statistically significant difference between the results of both methods (Mann-Whitney U test).

FIGURE 6. *Comparison of the manual and automated gating approach.* Comparison of the basophil activation level (% CD63 expression of identified basophils, **A**) and the number of basophils (**B**) for the manual and data-driven automatic gating (R analysis) using 1,376 individual samples (PBS controls, fMLP and oleosins (in different concentrations: 10,000, 1,000, 100, 10 ng/ml) from 51 study participants (21 peanut-allergic patients, 15 peanut-sensitized but non-allergic subjects, and 15 non-allergic individuals)). **C**, performance comparison of the manual and automated gating approach using a ROC-curve analysis for peanut oleosins (10,000 ng/ml concentration). **D**, illustration of the dose-response curves measuring the basophil activation level (% CD63 expression of identified basophils) for three peanut-allergic patients after application of the 12 antibody gating strategy, the three antibody manual gating strategy, and the three antibody automated gating strategy. Study subjects included in the respective experiments are listed in Table S1.

FIGURE 1:

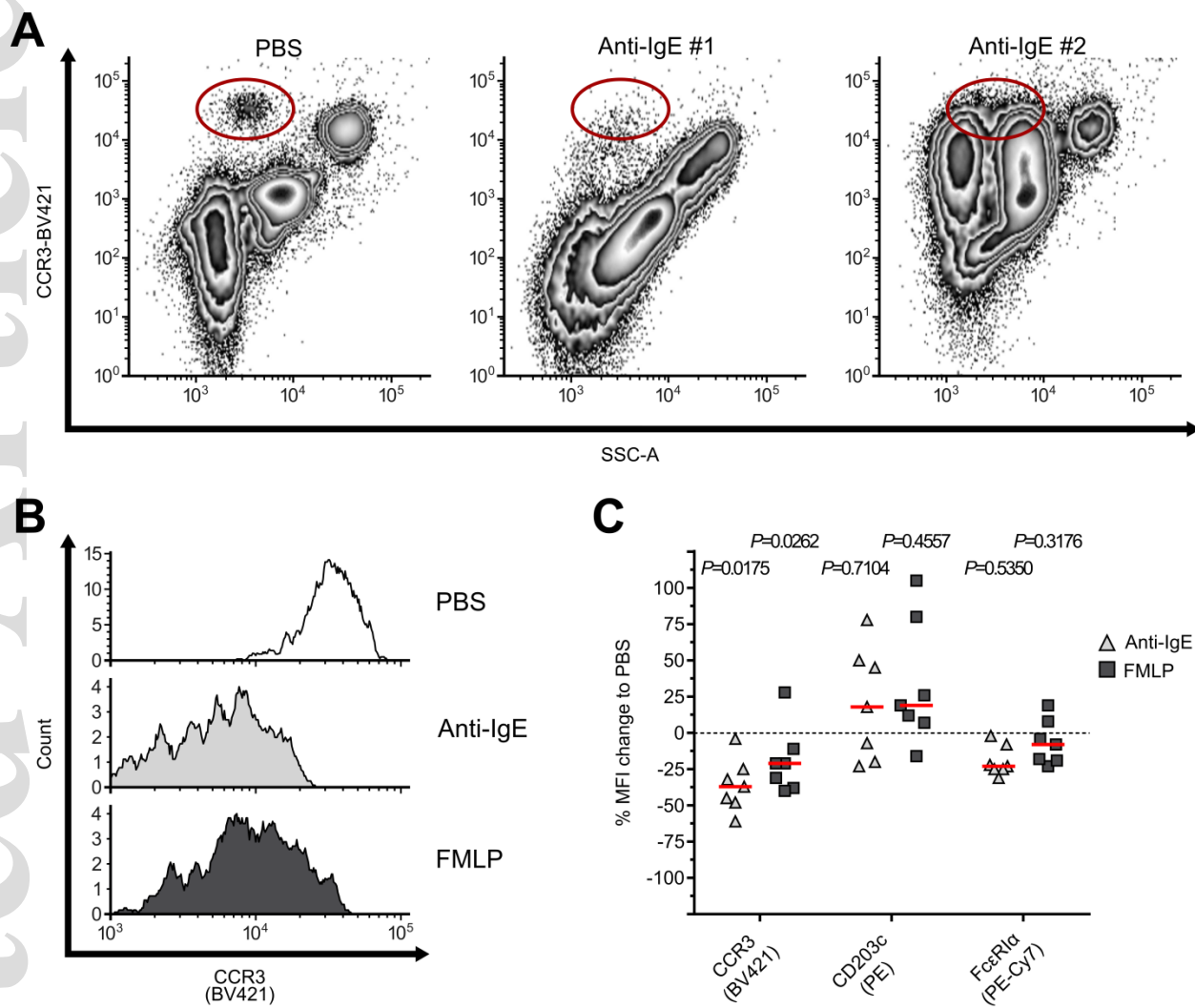


FIGURE 2:

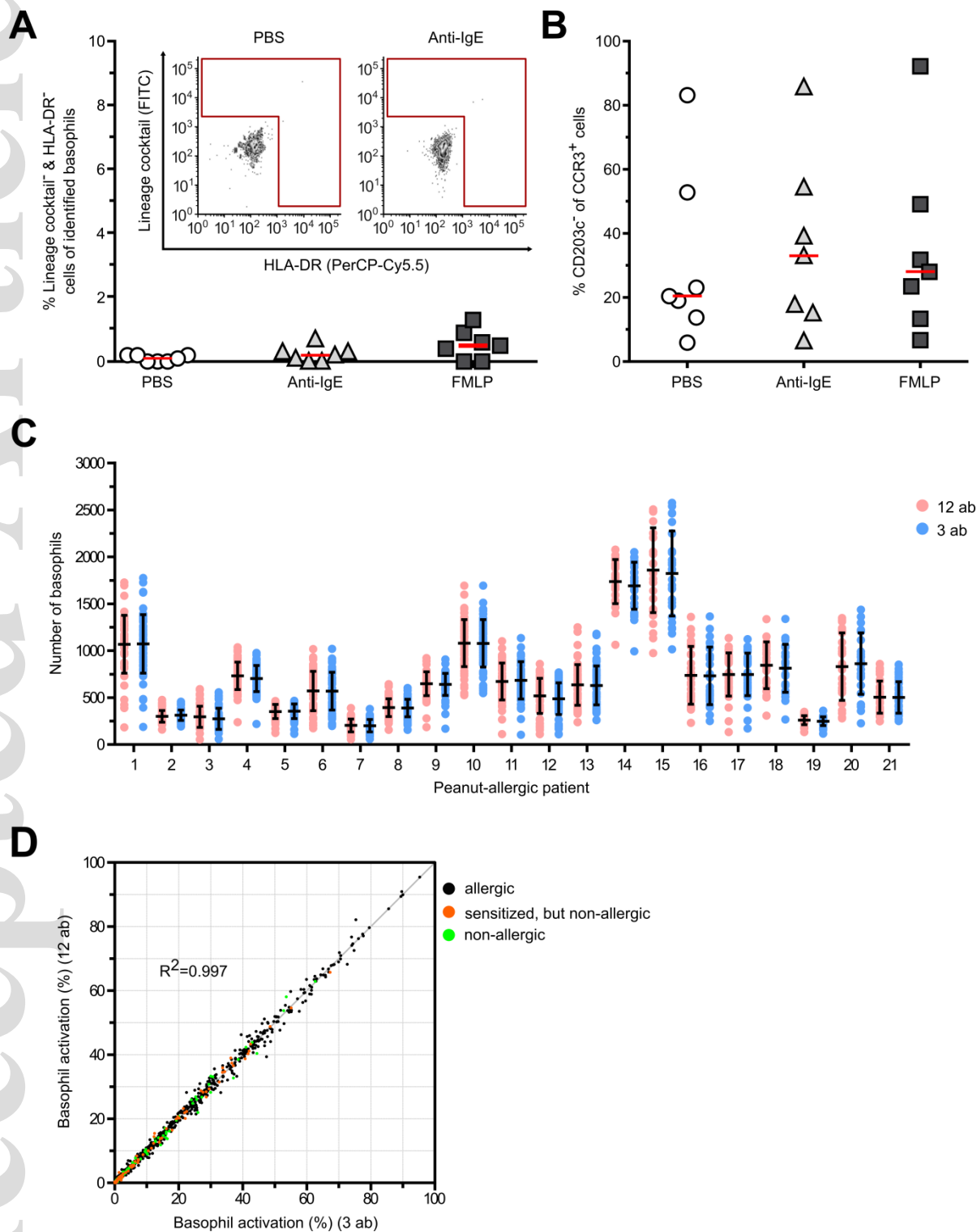


FIGURE 3:

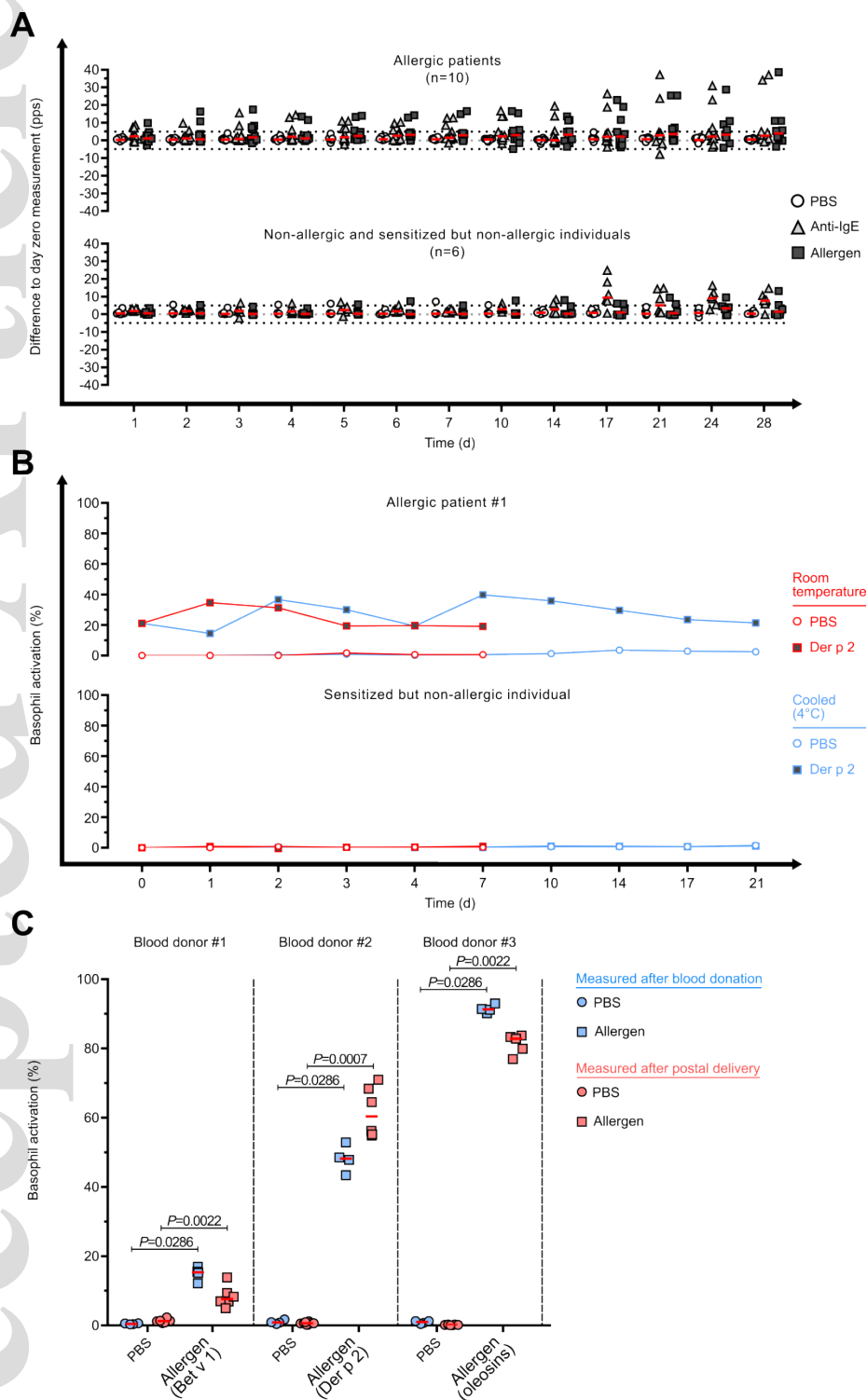
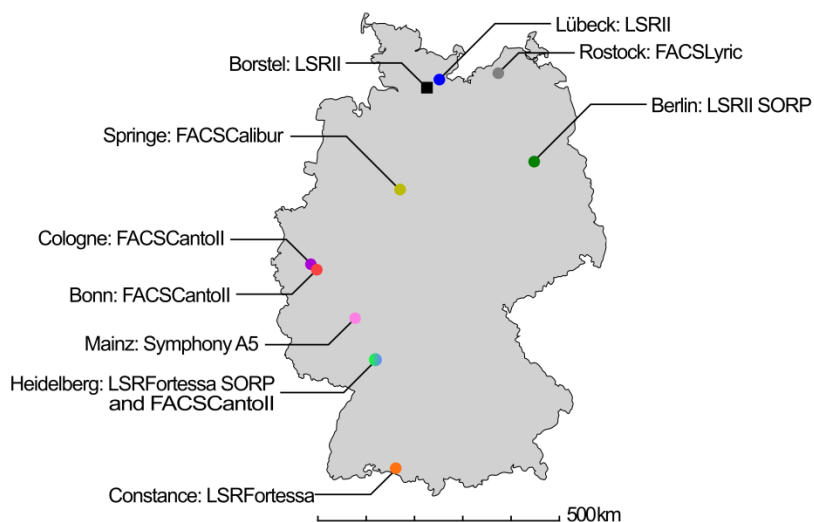


FIGURE 4:

A



B

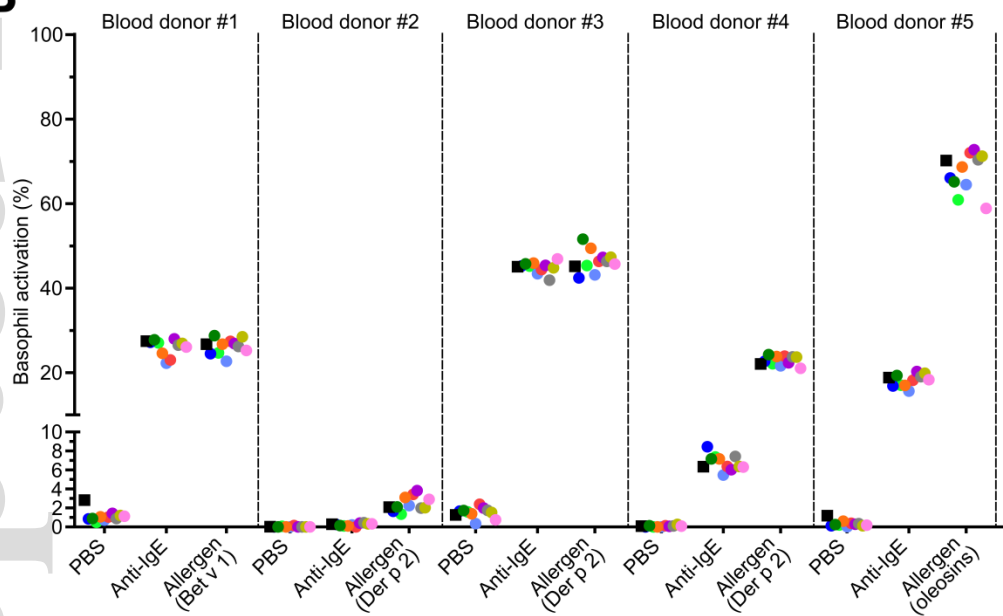


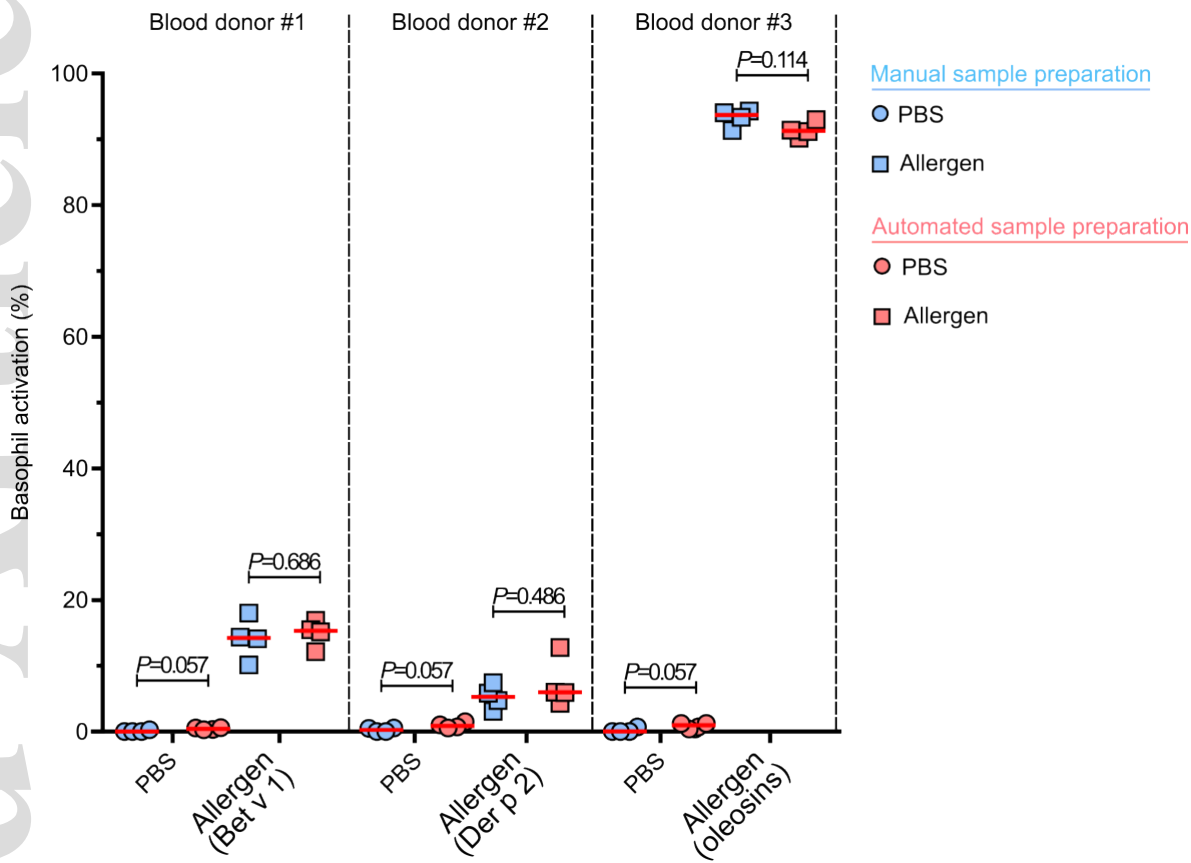
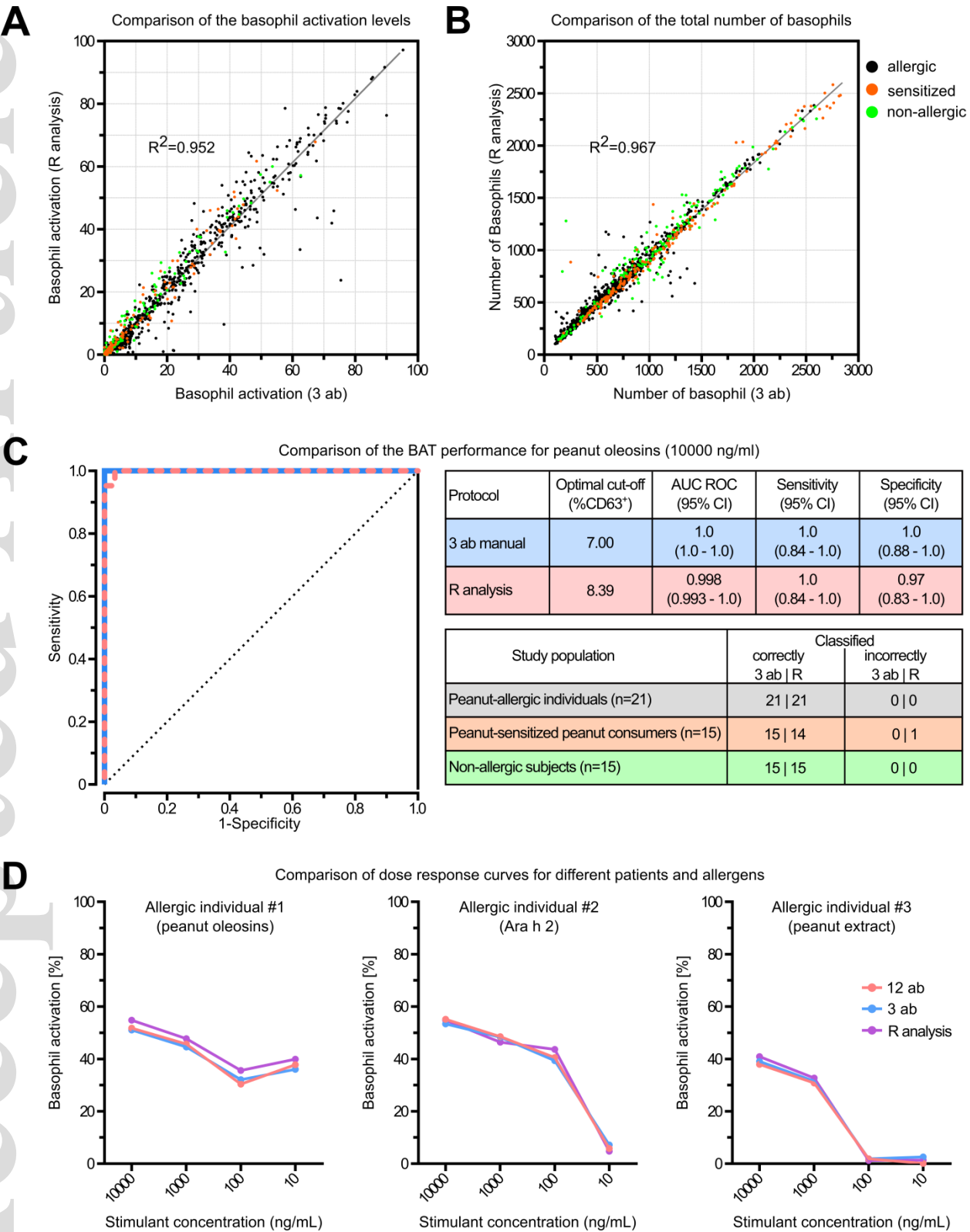
FIGURE 5:

FIGURE 6:



SUPPLEMENTARY APPENDIX

Innovative robust basophil activation test using a novel gating strategy for reliably diagnosing allergy with full automation

Jochen Behrends*, Christian Schwager*, Martina Hein, Thomas Scholzen, Skadi Kull, and Uta Jappe

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2 *Drs. Behrends and Schwager contributed equally to this article.

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SUPPLEMENTARY METHODS

Inter-laboratory survey

To access the feasibility of our method in daily praxis, an inter-laboratory survey was initiated and 9 further sites in Germany included (Lübeck, Rostock, Berlin, Springe, Mainz, Bonn, Heidelberg, Constance, Cologne). For the inter-laboratory survey, sampled blood from 5 allergic individuals was stimulated with the respective allergens (1x Bet v 1 (major allergen in birch), 3x Der p 2 (major allergen in the house dust mite *Dermatophagoides pteronyssinus*), 1x peanut oleosins), 5,000 ng/mL), anti-IgE and PBS and further prepared according to our standard protocol. The only exception was that the three antibody mixture was used instead of the 12 antibody cocktail. After sample preparation, samples were split and delivered to the destination site by the Deutsche Post AG (Bonn, Germany). Instruments at destination sites were set up using MACSQuant[®] Calibration Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and our defaults within the measured parameters (FSC, SSC, PE, PE-Cy7 and APC) with regard to MFIs of the beads within the fluorochrome channels before measurements (Protocol S1). Additionally, four thresholds using FSC, SSC, PE and PE-Cy7 as well as a stopping gate on basophils (800 events) had to be set by the laboratories. The FACSCalibur (Springe) was limited to two thresholds (FSC, PE-Cy7), because of the technical specifications of the instrument. Furthermore, samples were measured on two different flow cytometers in Heidelberg (intra-laboratory comparison). Sample measurements were performed between 3-7 days after blood sample work up. Data analysis was conducted in our laboratory with a fixed quadrant for the CD203c vs. CD63 gating and the calculation of the activation status of the basophils using % CD63⁺ cells of all basophils.

Evaluation of the BAT using an R script

Automatic analysis of the BAT was conducted with the statistical computing package R employing the flowCore package for the handling of flow cytometry data.^{1,2} Additional R libraries used include flowDensity, flowViz, flowStats, gridExtra, car, and caTools.³⁻⁸ For each individual several samples comprising different stimulants and controls were analyzed. The measurement of samples from multiple individuals has been evaluated during a single run. The R script first loads the flow cytometry data files in FCS3.0 format and transforms all values of the fluorescence channels and the side scatter using the estimateLogicle function. The gating of stimulated versus non-stimulated basophils is then performed in a three step process (Figure S5, I-III). First (I), doublet exclusion is achieved using a rectangular gate for singlet discrimination, the slope and position of which is set according to the linear regression of the FSC-A versus FSC-H parameters and the gate width to eight times the standard error of the regression. Second (II), the identification of the CD203c⁺/ FcεRIα⁺ positive basophil population is achieved by employing a custom 2D gating algorithm based on the sums of the expression values for both parameters. To avoid a bias due to an unequal scaling of the measured parameters, normalization is applied by forming a quotient of the 99.5% quantile of the highest expression values for FcεRIα and CD203c and subsequently multiplying the CD203c expression values by this correction factor. The sums of FcεRIα and normalized CD203c expression values are then calculated and stored in an R vector data structure. The elements of this vector are subsequently sorted from highest to lowest value. Two percent of events with the highest expression sums are then used to determine the basophil population. For this, the sorted expression values are grouped in 120 "bins" of equal width to generate a histogram representing the number of events in each group. The border of the basophil population is characterized by only few events in the

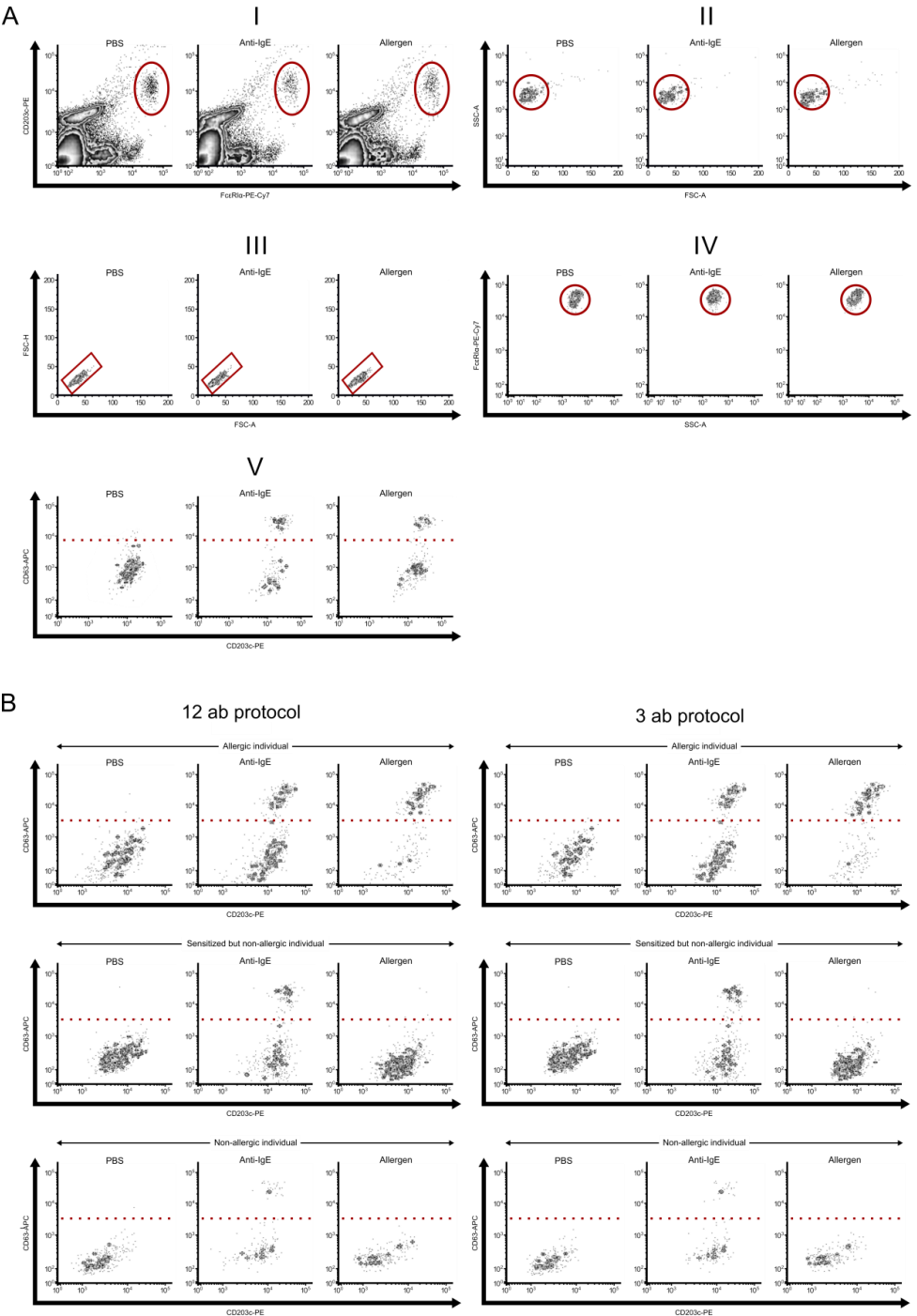
73 corresponding group (relative minimum) followed by a peak (relative maximum) in the center of
 74 the basophil population. For automatic gating all relative minima together with the highest
 75 subsequent maximum of the histogram values are identified. For each minimum/maximum pair
 76 the resulting ratio and the size of the corresponding hypothetical basophil population are
 77 estimated. To avoid the erroneous identification of abnormally small basophil populations
 78 consisting of only few events, a correction factor is introduced, favoring basophil populations of
 79 average size. The following correction factors were multiplied with the maximum to minimum
 80 ratio depending on the estimated hypothetical basophil fraction: hypothetical basophil fraction (f)
 81 $< 0.8\%$, correction factor (cf)=20; $0.8\% \leq f < 1.3\%$, cf=35; $1.3\% \leq f < 1.8\%$, cf=40; 1.8%
 82 $\leq f < 2.3\%$, cf=90; $2.3\% \leq f < 2.8\%$, cf=105; $2.8\% \leq f < 3.3\%$, cf=115; $3.3\% \leq f < 8.8\%$,
 83 cf=120; $8.8\% \leq f < 9.3\%$, cf=115; $9.3\% \leq f < 9.8\%$, cf=110; $9.8\% \leq f < 10.3\%$, cf=105;
 84 $10.3\% \leq f < 10.8\%$, cf=70; $10.8\% \leq f < 11.3\%$, cf=60; $11.3\% \leq f < 11.8\%$, cf=50; $f \geq$
 85 11.8% , cf=20. The histogram minimum corresponding to the largest value for the maximum to
 86 minimum ratio multiplied by the correction factor is chosen as the border of the basophil
 87 population. Therefore, all events exhibiting normalized CD203c/ FcεRIα expression sums equal
 88 to or higher than this histogram group are included in the preliminary basophil population. To
 89 remove outliers, this population is then additionally gated using the flowDensity function to
 90 apply an automatic 2D ellipsoid gate (position =c(T, NA), percentile =c(0.15, 0.15),
 91 use.percentile =c(True, True)) on the CD203c/ FcεRIα parameters to obtain the final basophil
 92 population. A large spread of the basophil population with regard to the CD203c/FcεRIα
 93 parameters is an indication of an erroneous identification of basophils. As a quality control a
 94 measure for the normalized spread of the basophil population is estimated for each sample. For
 95 this, the standard deviation of FcεRIα expression was multiplied by the standard deviation of

CD203c expression, divided by the mean of FcεRIα expression, divided by the mean of CD203c
 expression. Finally (III), the fraction of stimulated basophils is estimated using CD63 as an
 activation marker. Due to variations in the position of the unstimulated cells, the threshold is set
 considering unstimulated PBS controls as well as corrections for shifts in individual samples.
 First, for each individual the events of three unstimulated PBS controls are merged. To create a
 reference negative population from this merged control, outliers with high CD63 expression are
 removed using the automatic 1D deGate function from the flowDensity package (percentile =
 0.89, use.percentile = True). A general threshold (gT) is then calculated by adding 4.2 times the
 standard deviation to the mean of the reference negative population. To account for shifts in
 individual samples, the negative subpopulation for each sample is initially approximated by
 using the gT. The individual threshold (iT) for the samples is then calculated by adding 4.2 times
 the standard deviation to the mean of this negative subpopulation. The final threshold (fT)
 depends on the fraction of cells (F) below the iT and is calculated according to the formula: $fT =$
 $gT - F * (gT + iT) / 2$. For each individual the R script creates a report in PDF format including a
 graphical representation (visual quality control) of the gating steps for each sample – for a more
 convenient use in daily routine practice. This file also contains a tabular summary of the
 calculated values. In addition, the R script generates a spread sheet summarizing all results from
 the samples analyzed during the run. To minimize the effort of manual monitoring for
 plausibility of the generated results, the program displays automatic warnings that are given
 when (i) the total number of basophils is below 100, (ii) the basophil fraction has an unusual size
 (below 0.1% or above 2%), (iii) the number of total events is very small (below 10,000), (iv) the
 singlet fraction is below 80% (scatter problems), and/or (v) the basophil population exhibits a

118 large spread with regard to the FcεRIα and CD203c parameters (high variety of the expression
119 levels, above 0.011).

120 **SUPPLEMENTARY FIGURES**

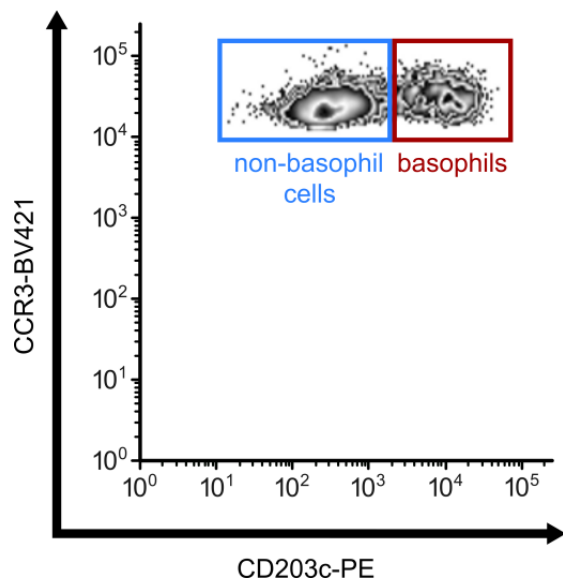
FIGURE S1. Detailed overview of our new two identification marker-based gating strategy combined with our three antibody protocol.



A, exemplary gating of basophils of a peanut-allergic patient stimulated with PBS, anti-IgE and peanut oleosins (allergen). (I) Identification of FcεRIα/CD203c positive basophils, (II) debris

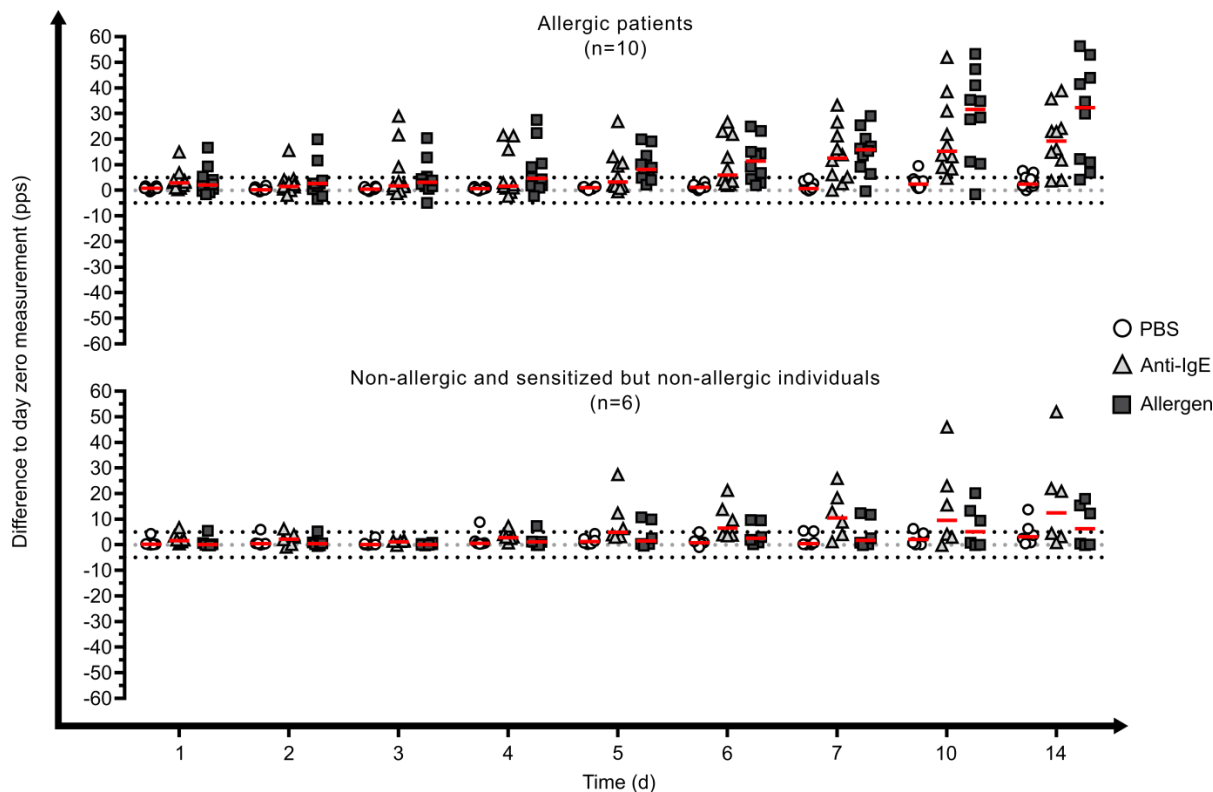
126 exclusion, (III) doublet exclusion, (IV) dendritic cells exclusion and (V) evaluation of the
127 basophil activation by CD63. **B**, a comparison of our 12 and three antibody protocol for
128 basophils from allergic, sensitized but non-allergic and non-allergic (healthy) individuals
129 showing PBS controls or activation upon anti-IgE and oleosins (allergen) stimulation. Study
130 subjects included in the respective experiments are listed in Table S1.

FIGURE S2. Analysis of the basophil purity using the CCR3 protocol.



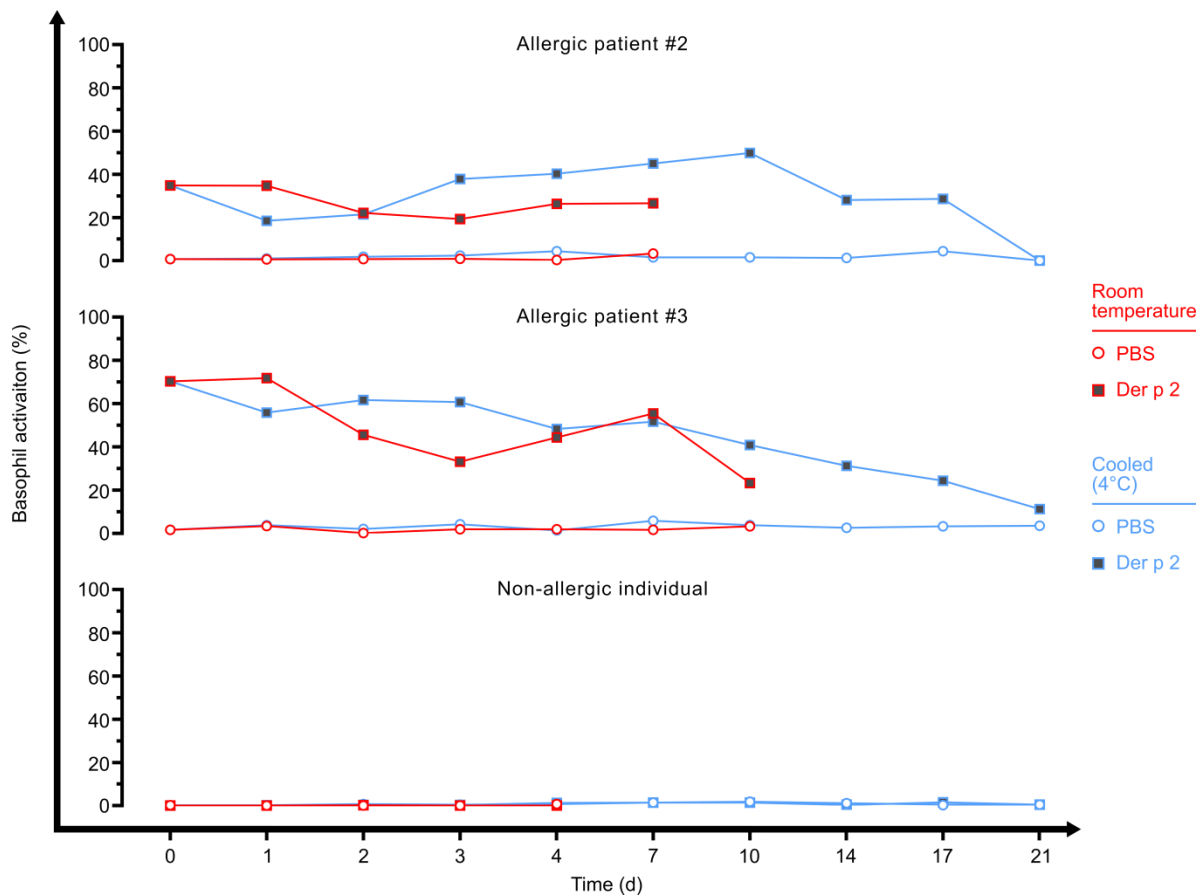
Gated basophils (Figure 1A, anti-IgE #2: CCR3^{high}/SSC^{low}) were further analyzed for CD203c expression to identify basophils (CD203c positive) and non-basophil cells (CD203c negative). Study subjects included in the respective experiments are listed in Table S1.

FIGURE S3. Evaluation of the BAT in prepared samples stored at room temperature.



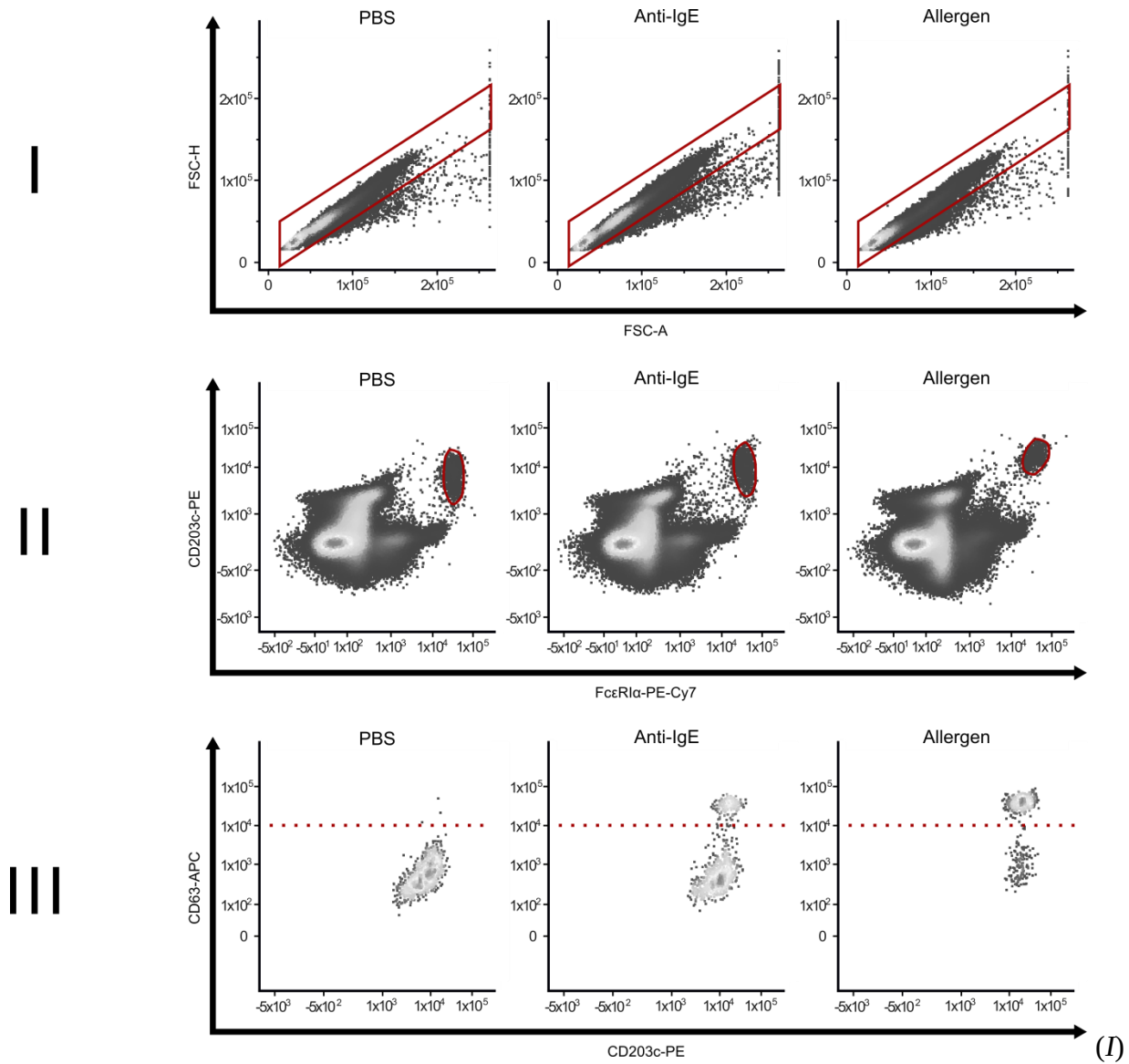
TS1: Progression of the basophil activation (data show changes (in percentage points) of activation (CD63⁺ basophils) in comparison to direct measurement at d0) for prepared samples stored at RT over a period of 14 days for 10 allergic patients (Bet v 1, Der p 2, oleosins; each in a concentration of 5,000 ng/ml) and six controls (three sensitized but non-allergic and three non-allergic (healthy) individuals). PBS: circle, anti-IgE: triangle, allergen: square. Study subjects included in the respective experiments are listed in Tables S1, S2, and S3. Red bars indicate medians of the groups.

FIGURE S4. Evaluation of the BAT in whole blood.



TS2: Progression of the basophil activation (% CD63⁺) for whole blood samples from two house dust mite-allergic patients and a non-allergic individual stored over a period of 21 days. Blood was stored at 4°C (blue) or RT (red) and prepared (with stimulation) for analysis at the given days. PBS: circle, Der p 2 (5,000 ng/ml): square. Study subjects included in the respective experiments are listed in Table S2.

152 **FIGURE S5.** Overview of the three antibody automated gating strategy (*R* analysis).



153

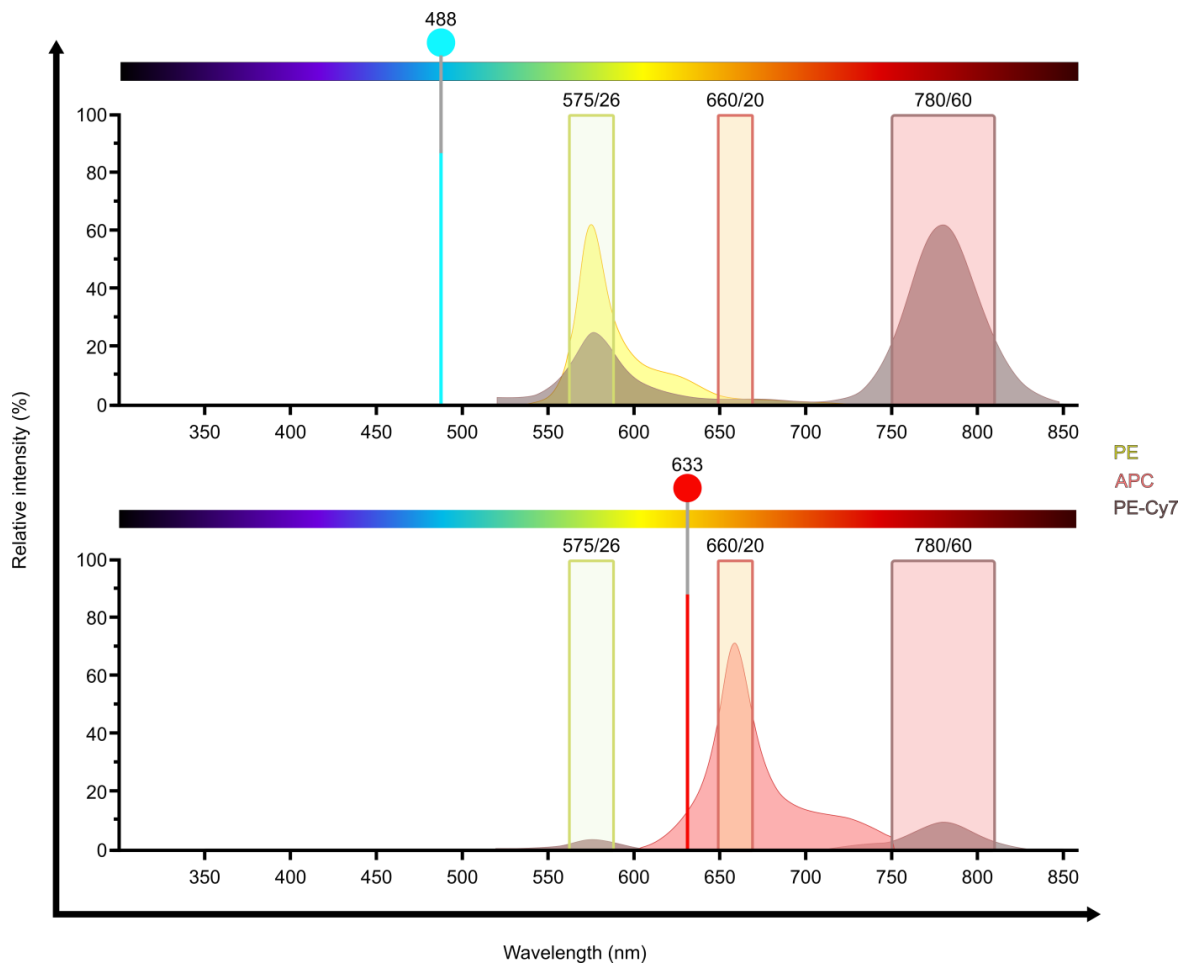
154 After a doublet exclusion (*I*, FSC-A vs. FSC-H), basophils are identified as (*II*)

155 CD203c⁺/FcεRIα⁺ cells and (*III*) analyzed for activation status by evaluation of the CD63⁺

156 fraction. The autogating algorithm performs an adjustment of the individual gates for each

157 sample. Study subjects included in the respective experiments are listed in Table S1.

FIGURE S6. Emission and detection range of the used fluorochromes.



Within the set-up of the three antibody protocol we choose the very bright fluorochromes PE (R-phycoerythrin), PE-Cy7 (Cyanine7®), and APC (Allophycocyanin). PE and PE-Cy7 are excited by the 488 nm Laser (blue dot and line). Their emission maximum ($E_{m_{max}}$) is at the wavelength of 578 nm (PE) or 778 nm (PE-Cy7). This $E_{m_{max}}$ is detected through bandpass (BP) filters 575/26 (collecting PE emission) and 780/60 (collecting PE-Cy7 emission). A time delayed second 633 nm Laser excited APC (red dot and line), and the emitted signal ($E_{m_{max}} = 660$ nm) is collected and detected through a 660/20 BP filter. No spillover effects of the fluorochromes PE and PE-Cy7 (used for the identification of the basophils: CD203c and FcεRIα) affecting the APC detector (readout channel for the activation of the basophils via CD63 expression).

170 **TABLE S1:** Demographic characteristics of study subjects included in BAT experiments with peanut allergens.

Patient	Sex	Age (y)	IgE ImmunoCAP (kU/L)								Western blot	Clinical characteristics	Data used in Figure
			Total IgE	Peanut extract	Ara h 1	Ara h 2	Ara h 3	Ara h 6	Ara h 8	Ara h 9	oleosins		
1	f	10	367.0	71.0	>100	41.0	24.7	>100	<0.35	0.5	pos.	Severe peanut allergy	2C/D, 6A-D, S5
2	m	19	732.0	>100	64.4	96.5	17.5	30.7	<0.35	<0.35	pos.	Severe peanut allergy	2C/D, 6A-D
3	m	32	536.0	75.9	21.4	51.0	1.2	20.9	6.1	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
4	m	18	1272.0	29.6	<0.35	6.6	<0.35	3.9	66.3	1.2	pos.	Severe peanut allergy	2C/D, 6A-D
5	f	26	271.0	5.1	<0.35	4.9	<0.35	3.5	0.4	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C, S1
6	f	20	1773.0	>100	87.0	>100	36.9	12.7	<0.35	<0.35	pos.	Severe peanut allergy	1A-C, 2A/B-D, 6A-C
7	m	22	92.4	25.4	15.6	11.7	3.7	6.6	<0.35	<0.35	pos.	Severe peanut allergy	2A/B-D, 3A/C, 4A, 5, 6A-C, S3
8	f	26	1616.0	>100	>100	>100	>100	9.6	<0.35	<0.35	pos.	Severe peanut allergy	1A/C, 2A/B-D, 6A-C
9	f	43	886.0	5.8	1.3	2.9	0.4	<0.35	14.3	1.0	pos.	Severe peanut allergy	2C/D, 6A-C
10	f	26	>2500	11.8	<0.35	40.3	0.4	18.4	18.3	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
11	M	23	1416.0	>100	69.6	34.0	13.1	17.9	9.9	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
12	m	15	628.0	>100	20.6	>100	48.9	68.7	<0.35	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
13	m	7	177.0	83.8	60.1	25.1	9.1	27.7	<0.35	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
14	f	43	793.1	5.8	2.4	1.2	0.7	<0.35	9.2	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
15	m	52	508.0	>100	51.1	60.5	9.0	40.4	17.3	4.9	pos.	Severe peanut allergy	2C/D, 6A-C
16	M	22	1267.0	>100	51.6	75.8	5.8	55.3	5.8	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
17	m	25	260.0	66.3	42.9	22.0	14.9	21.4	1.8	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
18	f	30	92.7	18.0	1.6	14.0	1.5	11.2	4.1	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
19	m	14	705.5	21.3	10.9	3.2	<0.35	1.3	<0.35	3.7	pos.	Severe peanut allergy	2C/D, 6A-C
20	m	11	430.5	>100	27.2	>100	1.0	94.5	<0.35	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
21	F	10	>2500	>100	>100	>100	47.2	>100	<0.35	<0.35	pos.	Severe peanut allergy	2C/D, 6A-C
22	F	19	343.4	87.1	77.5	14.8	1.7	3.78	<0.35	<0.35	pos.	Severe peanut allergy	1C, 2A/B
23	F	53	85.4	<0.35	<0.35	<0.35	<0.35	<0.35	2.5	0.5	neg.	Asymptomatic sensitization	2D, 6A-C

24	M	40	178.3	1.2	<0.35	<0.35	<0.35	<0.35	5.4	<0.35	neg.	Asymptomatic sensitization	1C, 2A/B, 2D, 6A-C
25	F	51	46.1	<0.35	<0.35	<0.35	<0.35	<0.35	1.4	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
26	F	42	222.0	0.9	<0.35	<0.35	<0.35	<0.35	3.2	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
27	F	36	146.5	<0.35	<0.35	<0.35	<0.35	<0.35	11.0	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
28	F	65	161.9	<0.35	<0.35	<0.35	<0.35	<0.35	4.8	0.4	neg.	Asymptomatic sensitization	2D, 6A-C, S1
29	F	36	111.5	<0.35	<0.35	<0.35	<0.35	<0.35	0.4	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
30	M	29	546	1.99	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
31	F	54	142	0.4	<0.35	<0.35	<0.35	<0.35	3.1	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
32	F	38	761.7	0.4	<0.35	<0.35	<0.35	<0.35	20.3	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
33	F	40	97.3	<0.35	<0.35	<0.35	<0.35	<0.35	1.3	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
34	F	24	385.4	<0.35	<0.35	<0.35	<0.35	<0.35	7.9	0.8	neg.	Asymptomatic sensitization	2D, 6A-C
35	F	25	240.7	<0.35	<0.35	<0.35	<0.35	<0.35	0.6	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
36	F	29	227.1	<0.35	<0.35	<0.35	<0.35	<0.35	0.6	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
37	F	50	62.8	<0.35	<0.35	<0.35	<0.35	<0.35	0.6	<0.35	neg.	Asymptomatic sensitization	2D, 6A-C
38	M	41	29.8	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
39	M	44	4.2	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
40	M	76	11.9	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
41	F	39	14.5	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
42	F	41	33.6	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
43	M	53	5.2	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
44	M	35	251.1	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
45	F	28	39.2	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C

46	F	41	7.7	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
47	M	47	61.4	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
48	M	36	21.9	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
49	F	35	53.6	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	1C, 2A/B, 2D, 6A-C, S1
50	F	49	9.4	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	1C, 2B
51	F	32	63.7	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	1C, 2A/B, 2D, 6A-C
52	F	29	54.1	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C
53	F	56	2.0	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	<0.35	neg.	Non-allergic	2D, 6A-C

171 **TABLE S2:** *Demographic characteristics of study subjects included in BAT experiments with house dust mite allergens.*

Patient	Sex	Age (y)	IgE ImmunoCAP (kU/L)						Clinical characteristics	Data used in Figure
			Total IgE	Der p extract	Der f extract	Der p 1	Der p 2	Der p 10		
1	M	31	230.4	12.3	23.1	2.5	8.9	<0.35	Allergic to house dust mite	3A, 3C, 4A, S3, S4
2	M	33	42.5	2.7	2.7	0.9	1.4	<0.35	Allergic to house dust mite	3A, S3
3	M	23	>2500	41.3	65.9	13.5	36.7	<0.35	Allergic to house dust mite	3A/B, 4A, S3
4	F	23	158.4	11.0	8.1	3.2	4.8	<0.35	Allergic to house dust mite	3A, 5, S3
5	M	40	178.3	5.4	5.3	1.4	4.3	<0.35	Allergic to house dust mite	3A, S3
6	F	40	97.3	5.9	7.4	2.3	3.9	<0.35	Allergic to house dust mite	3A, S3, S4
7	F	36	146.5	27.6	27.3	9.4	14.8	<0.35	Allergic to house dust mite	3A, 4A, S3
8	F	39	14.5	<0.35	<0.35	<0.35	<0.35	<0.35	Non-allergic	3A, S3
9	F	41	33.6	<0.35	<0.35	<0.35	<0.35	<0.35	Non-allergic	3A, S3, S4
10	M	76	11.9	<0.35	<0.35	<0.35	<0.35	<0.35	Non-allergic	3A, S3
11	M	29	546.0	<0.35	<0.35	<0.35	<0.35	<0.35	Allergic, but not to house dust mite	3A, S3
12	F	53	85.4	<0.35	<0.35	<0.35	<0.35	<0.35	Allergic, but not to house dust mite	3A/B, S3
13	M	30	22	<0.35	<0.35	<0.35	<0.35	<0.35	Allergic, but not to house dust mite	3A, S3

172 **TABLE S3:** *Demographic characteristics of study subjects included in BAT experiments with birch pollen allergens.*

Patient	Sex	Age (y)	IgE ImmunoCAP (kU/L)			Clinical characteristics	Data used in Figure
			Total IgE	Birch pollen extract	Bet v 1		
1	F	51	46.1	17.1	17.1	Allergic to birch pollen	3A, 4A, S3
2	M	31	162.8	43.5	41.5	Allergic to birch pollen	3A, S3
3	F	40	97.3	1.03	1.09	Allergic to birch pollen	3C, 5

173 **TABLE S4:** Location and technical specifications of flow cytometers involved in the ring trial.

Laboratory	Instrument	Laser (nm) FSC/SSC	Laser (nm)	Band Pass PE	Laser (nm)	Band Pass PE-Cy7	Laser (nm)	Band Pass APC
Flow & Mass Cytometry Core Facility Charité - Universitätsmedizin (Berlin)	BD™ LSR II SORP	488	561	582/15	561	780/60	635-40	660/13
Service Facility FACS & Microscopy Max Planck Institute for Biology of Ageing (Cologne)	BD™ FACSCanto II	488	488	585/42	488	780/60	633	660/20
FACS and Array Core Facility University Medical Center (Mainz)	BD™ Symphony A5	488	561	586/15	561	780/60	637	670/30
Cell Analysis Core Facility CAnaCore University of Lübeck (Lübeck)	BD™ LSR II	488	561	582/15	561	780/60	633	660/20
Flow Cytometry Core Facility German Center for Cancer Research (DKFZ) (Heidelberg)	BD LSRFortessa™ SORP	488	561	586/15	561	780/60	640	670/30
	BD™ FACSCanto II	488	488	584/42	488	780/60	640	670/14
Flow Cytometry Core Facility Medical Faculty University Bonn (Bonn)	BD™ FACSCanto II	488	488	585/42	488	780/60	633	660/20
FlowKon - Flow Cytometry Center University of Constance (Constance)	BD™ LSRFortessa	488	561	586/15	561	780/60	640	670/14
Center for Internal Medicine, Department of Pneumology, University of Rostock (Rostock)	BD™ FACSLyric	488	488	586/42	488	783/56	640	660/10

German Red Cross - Blood Donation Center NSTOB (Springe)	BD™ FACSCalibur	488	488	585/42	488	670LP	635	661/16
Core Facility Fluorescence Cytometry Research Center Borstel (Borstel)	BD™ LSR II	488	488	575/26	488	780/60	633	660/20

174 **TABLE S5.** Overview of anti-human antibodies used for BAT experiments.

Antibody	Clone	Fluorophore	Manufacturer
Anti-human CD45 [#]	HI30	BV510	Biolegend (Fell, Germany)
Anti-human CD63 ^{§, *, #}	H5C6	APC	Biolegend (Fell, Germany)
Anti-human CD193 (CCR3) [*]	5E8	BV421	Biolegend (Fell, Germany)
Anti-human CD123 [#]	6H6	BV421	Biolegend (Fell, Germany)
Anti-human CD203c ^{§, *, #}	NP4D6	PE	Biolegend (Fell, Germany)
Anti-human FcεRIα ^{§, *, #}	AER-37	PE-Cy7	Biolegend (Fell, Germany)
Anti-human HLA-DR [#]	L234	PerCP-Cy5.5	Biolegend (Fell, Germany)
Anti-human lineage cocktail [#]		FITC	Biolegend (Fell, Germany)
Anti-human CD3	UCHT1		
Anti-human CD14	HCD14		
Anti-human CD16	3G8		
Anti-human CD19	HIB19		
Anti-human CD20	2H7		
Anti-human CD56	HCD56		

175 [§] 3 antibody protocol, [#] 12 antibody protocol, ^{*} CCR3 protocol

SUPPLEMENTARY PROTOCOL

Protocol S1. Ring trial - Instructions for the BAT measurement.

Set-up flow cytometer – Part I:

Choice of parameters

lin FSC-A / H / W

log SSC-A / H / W

log PE-A

log PE/Cy7-A

log APC-A

Status	Parameters	Threshold	Laser	Compensation	Ratio
Parameter	Voltage	Log	A	H	W
FSC	500	☐	☒	☒	☒
SSC	200	☒	☒	☒	☒
PE	750	☒	☒	☐	☐
PE-Cy7	650	☒	☒	☐	☐
APC	420	☒	☒	☐	☐

Choice of thresholds:

FSC 20,000

SSC 500

PE 1,000

PE/Cy7 2,000

Status	Parameters	Threshold	Laser	Compensation	Ratio
☐ Or ☒ And					
Parameter			Value		
	FSC				20,000
	SSC				500
	PE				1,000
	PE-Cy7				2,000

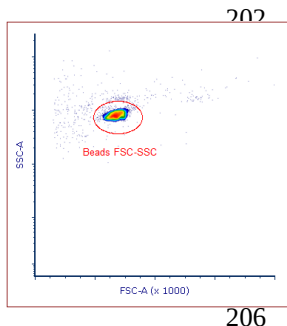
Set-up beads and samples:

Mix samples (“1-15”) and beads (“Beads”) by vortexing 2 times before opening; please check description carefully and transfer the samples as well as the beads from the Eppis to the FACS-tubes (volume: 500µl).

Set-up flow cytometer – Part II:

A: Put the “flow rate” of the flow cytometer on “low”, adjust voltages of the FSC-A (lin x-axis) / SSC-A (log y-axis) to locate the beads within a gate “Beads FSC-SSC” (picture below);

200 arithmetic mean intensities of the FSC and SSC should be as high as described below (if
201 necessary, adjust the voltages of the FSC and SSC):



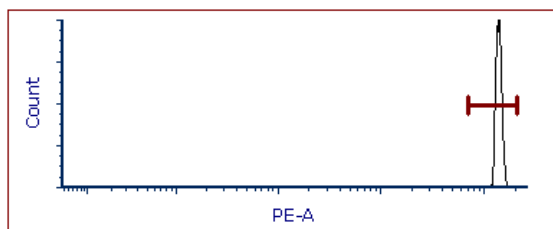
Bead position within the FSC-A/SSC-A dot plot:

lin FSC-A (arithmetic mean) = 90,000 ($\pm$ 3000)

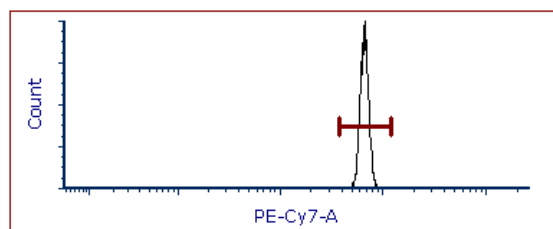
log SSC-A (arithmetic mean) = 8,400 ($\pm$ 1000)

207 **B:** Insert 3 histograms: PE-A, PE/Cy7-A and APC-A as subgates of “Beads FSC-SSC”; gate
208 positive peaks and adjust the arithmetic mean of the channels as described below:

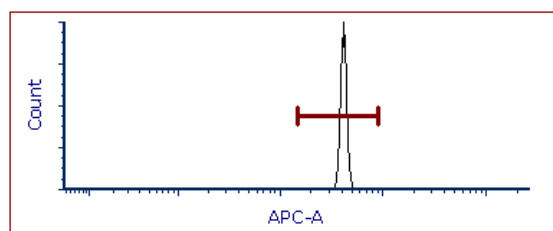
209 - Gate Beads FSC-SSC $\rightarrow$ PE-A signal (arithmetic mean) = 140,000 ($\pm$ 10,000)



214 - Gate Beads FSC-SSC $\rightarrow$ PE/Cy7-A signal (arithmetic mean) = 6,700 ($\pm$ 800)



220 - Gate Beads FSC-SSC $\rightarrow$ APC-A signal (arithmetic mean) = 4,200 ($\pm$ 600)

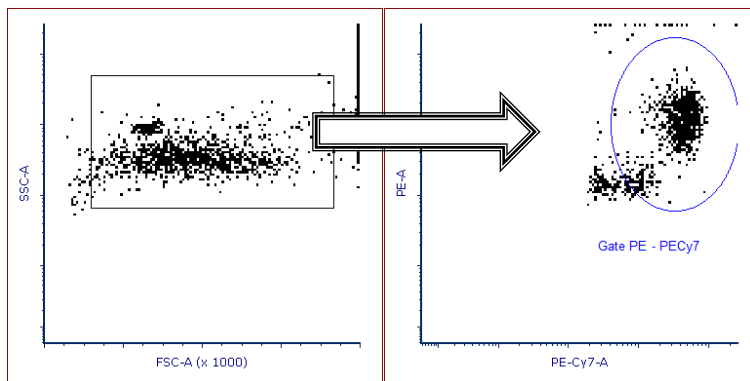


C: After the set-up of the beads following the instructions described within A and B, record 1,000 events within gate “Beads FSC-SSC” and name this sample “Beads”.

Sample measurement:

Samples are measured with a flow rate “high”. First measurement of all 15 samples is named 1A, 2A, ... The second measurement of all 15 samples is named 1B, 2B, ...

See figure below: within the FSC vs. SSC dot plot create a gate “basophils”, referring to this gate create a second dot plot PE vs. PE/Cy-7. Within this dot plot establish a gate “PE – PE/Cy7”. Use this gate as stop criterion if 800 events are recorded.



It is possible that during second measurements the event number in the stopping gate is below 800, because the sample is running out. If so, just measure until the sample is empty and record the data.

Data export:

Export data as FCS files 3.0.

Files can be transferred via email or WeTransfer (<https://wetransfer.com/>).

SUPPLEMENTARY REFERENCES

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