Microfluidic Immunoaffinity Basophil Activation Test for Point-of-Care Allergy Diagnosis

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Background: The flow cytometry-based basophil activation test (BAT) is used for the diagnosis of allergic response. However, flow cytometry is time-consuming, requiring skilled personnel and cumbersome processing, which has limited its use in the clinic. Here, we introduce a novel microfluidic-based immunoaffinity BAT (miBAT) method.

Methods: The microfluidic device, coated with anti-CD203c, was designed to capture basophils directly from whole blood. The captured basophils are activated by anti-FcεRI antibody followed by optical detection of CD63 expression (degranulation marker). The device was first characterized using a basophil cell line followed by whole blood experiments. We evaluated the device with ex vivo stimulation of basophils in whole blood from healthy controls and patients with allergies and compared it with flow cytometry.

Results: The microfluidic device was capable of capturing basophils directly from whole blood followed by in vitro activation and quantification of CD63 expression. CD63 expression was significantly higher ($P = 0.0002$) in on-chip activated basophils compared with nonactivated cells. The difference in CD63 expression on anti-FcεRI-activated captured basophils in microfluidic chip was significantly higher ($P = 0.03$) in patients with allergies compared with healthy controls, and the results were comparable with flow cytometry analysis ($P = 0.04$). Furthermore, there was no significant difference of CD63% expression in anti-FcεRI-activated captured basophils in microfluidic chip compared with flow cytometry.

Conclusions: We report on the miBAT. This device is capable of isolating basophils directly from whole blood for on-chip activation and detection. The new miBAT method awaits validation in larger patient populations to assess performance in diagnosis and monitoring of patients with allergies at the point of care.

IMPACT STATEMENT

Asthma and allergic diseases are common health problems in the Western world, affecting up to 30% of the population. Basophils are a key player in the allergic reactions, and new biological pharmaceuticals that target basophil function have been developed. A method for an accurate point-of-care test of basophil function is potentially helpful. Here, we present a novel microfluidic-based point-of-care test of basophil activation. The chip is optimized to capture viable basophils directly from whole blood for on-chip activation and detection. Our technology could potentially facilitate diagnosis and monitoring of allergic responses in patients at the point of care.

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Peripheral blood is the most frequently accessed biological material in the clinic, and isolation of cells from blood is of broad clinical and scientific importance. Leukocytes are responsible for providing immunity (*1*, *2*) but also have an important role in the pathogenesis of inflammatory diseases such as allergic reactions. Basophils represent a subpopulation of leukocytes constituting <1% of the total peripheral circulating leukocytes (*3*, *4*). Basophils are involved in the inflammatory responses of allergic reactions, mainly IgE-mediated allergic reactions that are provoked by reexposure to a specific allergen. The IgE antibodies (IgE-ab)[3] produced by activated plasma cells predominantly bind to specific IgE receptors (FcεRI) on the surface of mast cells and basophils. Cross-linking of the allergen to IgE-ab on basophils causes activation, degranulation, and release of a variety of immune modulators such as histamine (*5*, *6*). Histamine promotes increase of vascular permeability and smooth muscle contraction that may proceed into a fatal clinical systemic condition (anaphylaxis) (*7*).

Allergy is a worldwide medical problem. The prevalence of allergic reaction is estimated to be 25% to 30% (*8*), and the rate of mainly food allergy is increasing, especially in young people. Understanding mechanisms and patient-specific risk factors constitutes the key to improve the diagnostics, monitoring, and treatment of individuals who have susceptibility to developing an allergic reaction. Diagnosis of allergy has mainly relied on patient history, analysis of IgE-ab, and/or skin prick test to the allergen in question. To achieve a more reliable diagnosis, in vivo challenges can be performed. However, in vivo challenge tests are less specific, less sensitive, and always mean a risk for the patient to develop a severe allergic reaction (*9*, *10*). The discovery of CD203c as a specific biomarker for basophils (*11*, *12*) has improved the investigation techniques of basophil activation using flow cytometry. The basophil activation test (BAT) is a method used in the clinic to measure the expression of activator markers on the basophil surface such as CD63 and CD203c (*13*). CD63 is a degranulation marker present in the inner surface of cytoplasmic granules and becomes exposed on the surface following granule exocytosis and can thereafter be detected by flow cytometry (*14*, *15*). BAT has been evaluated as a method for allergy diagnosis in the clinic, and there has been general agreement between the clinical presentation (systemic reaction vs large local reaction) and the results of BAT, suggesting that the BAT is a potential biomarker of allergy (*13*). The BAT can also be used to monitor patients undergoing allergen immunotherapy (*16*) and treatment with anti-IgE (omalizumab) by performing basophil allergen threshold sensitivity (CD-sens). CD-sens has been shown to correlate significantly with IgE-ab measurements, skin prick test, and nasal, bronchial, and oral allergen challenges (*17*). However, the cost, technical requirements for the operation and maintenance of flow cytometry as a technique for BAT, and the cumbersome preprocessing and prelabeling of the sample before flow cytometric analysis have limited its clinical application.

The need for competent cell-based diagnostic tools for various diseases has led to the development of novel microfluidic separation techniques. Microfluidics offer numerous advantages, including reduced sample volumes, faster sample processing, high sensitivity, low cost, and increased

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[3] **Nonstandard abbreviations:** IgE-ab, IgE antibodies; BAT, basophil activation test; miBAT, microfluidic-based immunoaffinity approach; PDMS, polydimethylsiloxane; MFI, mean fluorescence intensity; PE, phycoerythin.

portability (*18*, *19*). To date, many microfluidic cell isolation technologies have been developed using immunoaffinity (*20*), size (*21*), and electrical properties for separation (*22*). Among these techniques, immunoaffinity provides a more specific method for cell enrichment, in which antibodies against the cells of interest are immobilized onto the microfluidic surface for cell capture followed by optical detection (*18*, *23*). Although affinity capture has been successfully used to capture leukocyte subpopulations such as neutrophils (*24*) and lymphocytes (*23*) directly from whole blood, to the best of our knowledge, the specific on-chip capture of basophils from whole blood has not been reported. The FcεRI signaling pathway, which is responsible for triggering allergic reactions, has been studied in a continuous flow microfluidic platform using cultured RBL-2H3 cells (*25*). It was also recently reported that the activation of basophil cell lines (KU812 cells) was investigated using an integrated centrifugal microfluidic platform to screen agents that can block allergic activities (*26*, *27*). However, the basophil cell line (KU812) is a human leukemia cell line. Cancer cells undergo a variety of changes that will interfere with cell function and surface marker expression as compared with healthy cells; therefore, they cannot be considered equivalent to basophils (*28*).

Here, we report on a microfluidic-based immunoaffinity approach (miBAT) that rapidly isolates basophils directly from the patient's blood to diagnose allergy and compare it with the established fluorescence-activated cell sorter-based BAT assay. The microfluidic device captures CD203c-positive cells in a single step directly from whole blood without prelabeling and preprocessing of the sample. The captured cells are then activated, which triggers the exposure of the CD63 to the surface of the cells and can be measured. To validate the method, we perform a BAT after capturing the cells using anti-FcεRI antibody, followed by detection of CD63 using fluorescent microscopy, and then compare the results with the established flow cytometry BAT.

MATERIALS AND METHODS

Device microfabrication

We have used a microfluidic device design that has a straight channel, in which the width, height, and length of the channel were 4 mm, 50 µm, and 25 mm, respectively. The microfluidic device was fabricated in polydimethylsiloxane (PDMS) using standard soft lithography techniques (*29*). Briefly, channel replicas were produced using a negative photoresist SU-8 (MicroChem) onto the silicon wafer using standard microelectromechanical systems technology. The height of the SU-8 pattern on the master was measured using a surface profilometer. The devices were produced by casting PDMS onto the patterned silicon wafers. The elastomeric PDMS (Dow Corning) was mixed with a cross-linker at a ratio of 10:1 (wt/wt) and poured onto the master, degassed, and cured at 65 °C for 6 h. The curved PDMS with replicated channels was peeled off from the silicon wafer, and channel holes were punched with a Harris Uni-Core™, Tip ID 0.75 mm. The PDMS replica was bonded to a glass slide after brief oxygen plasma treatment.

Surface modification

3-Mercaptopropyl trimethoxysilane (Sigma Aldrich) was infused and incubated for 1 h. This was followed by washing with ethanol and the addition of 4-maleimidobutyric acid *N*-hydroxysuccinimide ester (Sigma Aldrich), a cross-linking agent, for 30 min. The devices were washed again first with ethanol and then with PBS. Neutravidin (Sigma Aldrich) was added, and the devices were stored at 4 °C. Before the experiments, the devices were incubated with biotinylated anti-CD203c (MACS, Miltenyi Biotech) overnight.

Blood sampling

Venous blood samples from healthy donors (n = 8) (Blood Bank, Stockholm, Sweden) and patients with allergies (n = 8) (Sachs's Children and Youth Hospital, Stockholm, Sweden) were collected in 10-mL Na^+ Heparin Vacutainer Tubes (Becton Dickinson) and analyzed within 3 h. The study was approved by the regional ethics committee in Stockholm, Sweden (Dnr. 2014/1630–31/4).

Cell line KU812 culture

The KU812 cell line (basophil cell line) samples were cultured in RPMI-1640 media containing 10% fetal bovine serum and 0.2% nonessential amino acids (Sigma Aldrich). Cells were cultured in a CO_2 incubator, and the medium was renewed every 2 to 3 days through standard cell culture practice.

Cell capture in microfluidic chip

KU812 basophil cells were washed and resuspended in 1× PBS for processing into the chip. The devices were washed with 1% BSA in 1× PBS, pH 7.2, at 20 µL/min to wash out the unbound antibody. Then 70 µL of the sample was pumped into the straight channel at flow rates of 1 to 20 µL/min using a syringe pump (Harvard Apparatus PHD 2000). The chips were washed with 1% BSA (w/v) at 20 µL/min for 10 min to remove the unbound cells. Captured cells were stained using nuclear staining (Hoechst stain) (Sigma Aldrich), and fluorescent images were taken by fluorescent microscope; 3 measurements were made, corresponding to three 1-mm^2 squares in that vicinity. For the whole blood experiment, the sample was introduced into chip at flow rates of 3 to 10 µL/min, and the chips were washed with 1% BSA at a flow rate of 20 µL/min. The captured cells from whole blood were stained using nuclear staining (Hoechst stain). In addition, CD203c was used to stain captured basophils in chip. Cells were fixed by 4% paraformaldehyde for 10 min at room temperature, followed by washing of chip. Captured cells were incubated with anti-CD203c (Abcam) for 1 h at room temperature, followed by conjugation of primary antibody with fluorescently conjugated phycoerythrin (PE) antimouse secondary antibody (Abcam). Finally, the chips were visualized by Eclipse Ti Nikon microscope, images were acquired by Zyla 5.5 sCMOS Andor camera, and images were processed using ImageJ software.

Depletion experiments

The flow cytometry experiments were performed to estimate the capturing efficiency and purity of basophils from whole blood. Depletion assays were done by counting basophil (CD203c) cells in the samples collected before and after the passage through the microfluidic device. Samples were incubated with CD203c-PE (Abcam) for 25 min at 4 °C. Thereafter, lysis of erythrocytes was carried out with cold isotonic solution (154 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$ supplemented with 0.1 mmol/L EDTA, pH 7.2), and samples were centrifuged for 5 min at 300*g* at 4 °C. Cells were washed once with PBS and resuspended in 300 µL of cold PBS; flow count beads (Beckman Coulter) were added to calculate the absolute number of basophils and leukocytes in the outlet aliquots using flow cytometry.

Activation of basophils

Activation of basophils in microfluidic chip. The on-chip captured basophils were activated with anti-FcεRI antibody. Then 3 µg/mL anti-FcεRI was added into the chips and incubated at 37 °C for 20 min in a humidified chamber. Chips were washed with 1% BSA. Cells were fixed and incubated for 30 min at room temperature with CD63 Alexa-647 (Abcam). Finally, chips were washed with 1% BSA and imaged by fluorescent microscope. The experiments were performed with samples from healthy donors and patients with allergies.

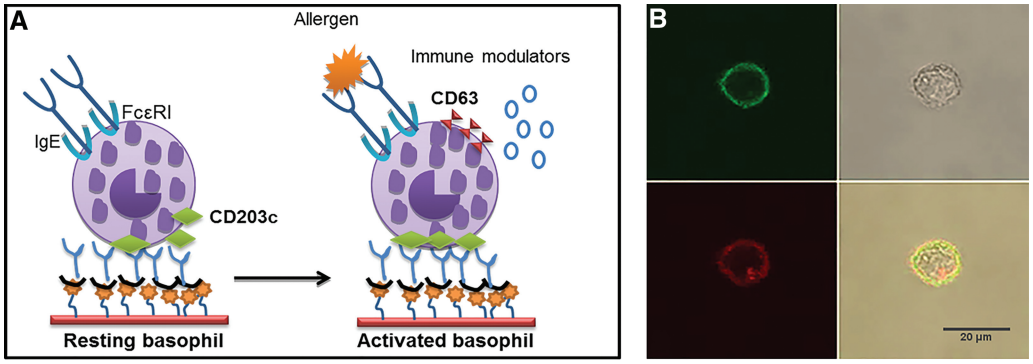

Fig. 1. Schematic illustration of the surface functionality of the microfluidic device and activation of captured basophil.
(A), The activation of captured basophils after the cross-link of the FcεRI-IgE complex, and the release of mediators from basophil granules leading to upregulation of CD203c and CD63 on the basophil surface. (B), Fluorescent signal on the surface of a basophil cell line after activation (on glass slide). The green signal shows CD203c and the red signal shows CD63. Scale bar: 10 µm.

Flow cytometric analyses of basophil activation. Flow cytometry analysis of CD63 and CD203c expression was performed, after activation with anti-FcεRI (Bühlmann Laboratories) of whole blood from healthy individuals and patients with allergies. RPMI (Sigma Aldrich) was used as negative control. Thereafter, the samples were stained with anti-CD203c-PE and anti-CD63 FITC (Beckman Coulter) and analyzed by flow cytometry.

Flow cytometry analysis

Flow cytometric analyses of basophils were done with a Navios flow cytometer (Beckman Coulter). Data were analyzed by Kaluza Analysis Software (Beckman Coulter).

Basophils were gated according to their granularity on side scatter and expression of CD203c. More than 200 basophils were analyzed on each run.

Statistical analysis

Scatter plots were prepared by GraphPad Prism 5, representing the range with whiskers and a middle line as the median. Statistical analysis was done with GraphPad Prism 5. Significant differences between groups were analyzed using the post hoc Mann–Whitney test. A P value of <0.05 was considered significant.

RESULTS

The principle of the BAT is shown in Fig. 1A. Conventionally, flow cytometry has been used for detection and quantification of basophil activation markers. As can be seen in Fig. 1B, basophil activation level is possible to measure using fluorescent microscopy. In this work, we have developed a miBAT assay. Below, we first describe the chip design and characterization, followed by basophil isolation and on-chip activation experiments from healthy individuals and allergy patients.

Microfluidic chip design and characterization

We designed and characterized a device for capturing basophils directly from whole blood. The surface of the microfluidic device was coated with anti-CD203c antibody to capture basophils.

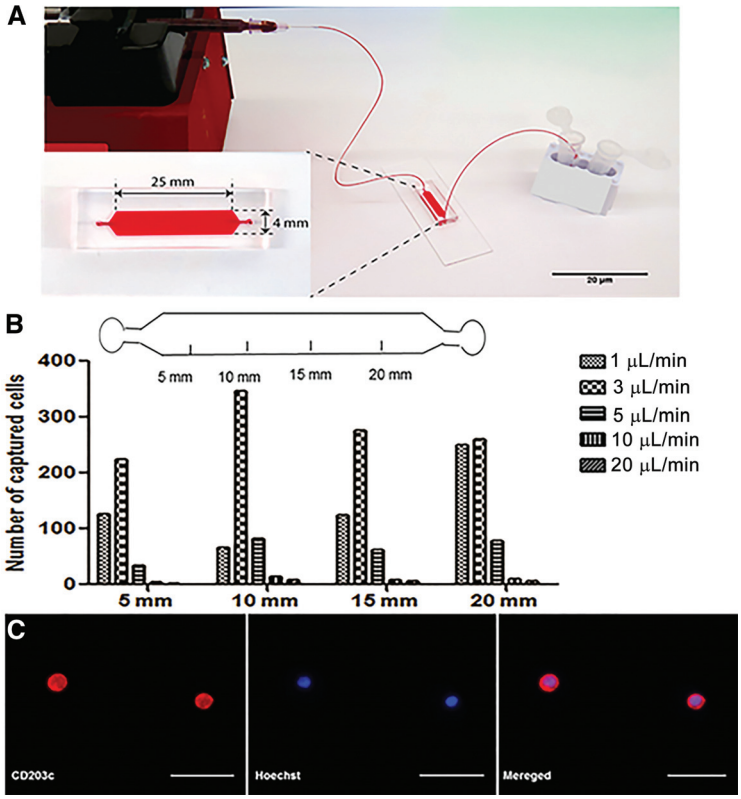

Fig. 2. Microfluidic setup and device characterization.
(A), The setup consists of a syringe pump connected to the microfluidic device. The enlarged box shows the dimensions of the 25-mm-long and 50-µm × 4-mm (height × width) device with a total internal volume of 5 µL. (B), Basophil cell line capture as a function of flow rate at different positions (5 mm, 10 mm, 1mm, and 20 mm) in the chip. (C), Fluorescently stained captured basophils in the functionalized chip. Scale bar: 50 µm.

Initially, an analysis of cell capture over a range of shear stresses for the cell capture and washing flow rates was conducted using a basophil cell line (KU812). The optimal flow rate using the straight channel (50 µm × 4 mm height and width) corresponded to 3 µL/min (Fig. 2). Fig. 2A shows the microfluidic device connected to a syringe pump, and Fig. 2B shows the cell capture as a function of the flow rate. Once captured, the cells can withstand a higher washing flow rate. Here, we used 20 µL/min for the washing step. The captured cell coverage is relatively uniform over the width of the channel, while there is a difference in the cell capture along the length of the channel where the maximum adhesion of cells was at 10 mm of chip length. The captured cells were stained with anti-CD203c fluorescent conjugated antibody to count the number of CD203c-positive cells as shown in Fig. 2C. Moreover, using control chips (without anti-CD203c coating), we were able to confirm that the capture is specific. Based on these experiments, we decided to examine the basophil capture directly from whole blood.

Basophil isolation from whole blood

To investigate immunoaffinity capture of basophils from whole blood, 200 µL of whole blood was

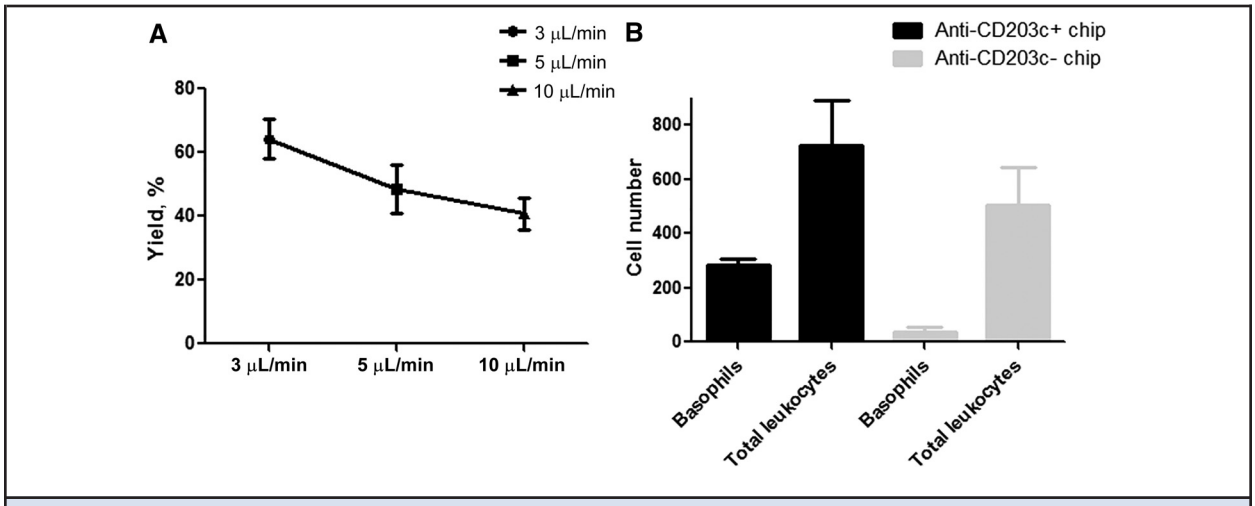

Fig. 3. Basophil capture from whole blood.
(A), Cell capture yield at different flow rates (3–10 μL/min). An optimal flow rate of 3 μL/min results in a yield of 64% basophil cell isolation from whole blood (n = 3). (B), The purity of captured basophils, calculated by the ratio (number of captured basophils/total number of leukocytes) in the chip, was 40% (n = 3). Most of the basophil cells were specific captured (see CD203c-negative chip).

processed through the channel using a syringe pump. As can be seen in Fig. 3A, the highest basophil capture yield was obtained for a flow rate of 3 μL/min, which is in agreement with the cell line-based results. When the flow rate was increased to 5 μL/min, the yield decreased from 64% to 49%. The optimal flow rate of 3 μL/min was chosen for all subsequent experiments. The yield was analyzed by flow cytometric analysis of the blood basophil cell counts before and after flowing of blood sample through the chip channel. Next, on-chip imaging was performed to fully characterize the microfluidic-based affinity capture in terms of purity and specificity of the antibody (Fig. 3B). The purity, calculated as the ratio of the CD203-positive cells to total leukocyte, was 40% (Fig. 3B).

Basophil expression of CD203c in healthy donors and allergy patients

To evaluate the miBAT assay, the captured basophils from whole blood were stimulated by anti-FcεRI antibody to induce degranulation of basophils, and RPMI was used as negative control. This was followed by detection of CD203c expression using fluorescent microscopy (Fig. 4A) and flow cytometry (Fig. 4B). We observed that the CD203c mean fluorescence intensity (MFI) in captured activated basophils was significantly higher than the negative control (nonactivated basophils) in healthy individuals ($P = 0.02$) and allergy patients ($P = 0.04$) (Fig. 4A). Comparable results were obtained using flow cytometry for both healthy donors and allergy patients ($P = 0.0002$) (Fig. 4B).

Basophil expression of CD63 in healthy donors and allergy patients

Expression of CD63 (degranulation marker) was analyzed in basophils captured in the microfluidic device. CD63% expression was significantly higher in anti-FcεRI-activated basophils (median, 45%; range, 36%–69%) as compared with nonactivated basophils from healthy controls (median, 22%; range, 10%–33%; $P = 0.0002$). A similar difference between allergy patients and healthy controls was obtained with the flow cytometry analysis

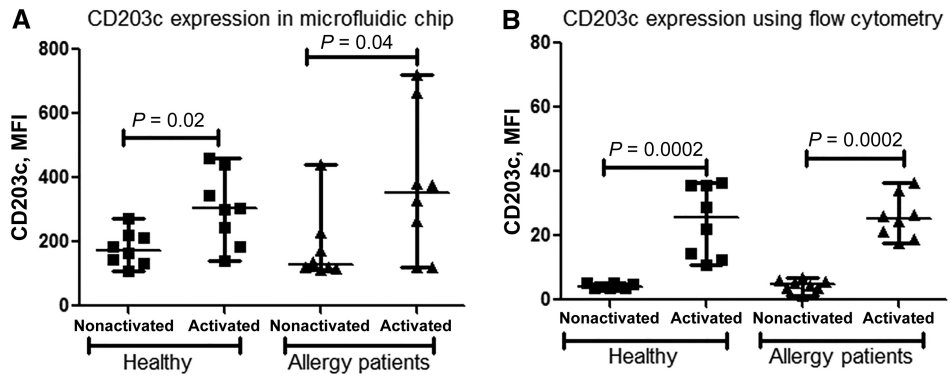

Fig. 4. Basophil expression of CD203c MFI in healthy donors and allergy patients.
(A), CD203c expression of anti-FcεRI-activated captured basophils compared with nonactivated cells in microfluidic chip in healthy controls compared with allergy patients. Scatter plots representing the range with whiskers and a line as the median (n = 8). (B), Flow cytometry analysis of CD203c MFI in activated basophils compared with negative control in allergy patients and healthy individuals (n = 8). A *P* value of <0.05 was considered significant.

(P = 0.0009) (Fig. 5A). To further investigate the difference in basophil activation between background and stimulated cells, we performed further analysis of CD63 expression level in basophils activated with anti-FcεRI and compared with nonactivated captured basophils. The difference in CD63 MFI at the single-cell level was significantly higher (P > 0.0001) in anti-FcεRI-activated basophils than in nonactivated captured cells (Fig. 5B). The CD63 MFI ratio (MFI of anti-FcεRI-activated/nonactivated basophil) was 2.4.

Moreover, the difference of CD63 expression was significantly higher in allergy patients compared with healthy controls in microfluidic chip (P = 0.04) (Fig. 6A). The result was comparable with flow cytometry analysis of CD63 expression comparing both groups (P = 0.03) (Fig. 6B). Furthermore, there was no significant difference of CD63% expression in anti-FcεRI-activated captured basophils in microfluidic chip compared with flow cytometry (Fig. 6C).

DISCUSSION

In this study, for the first time we introduced microfluidic-based basophil isolation from whole blood for miniaturized BAT analysis. We demonstrated that the CD63 in anti-FcεRI-activated captured basophils in allergy patients was significantly higher compared with nonactivated basophils (negative control). We also report that CD63 expression on activated captured basophils in microfluidic chip is significantly higher in allergy patients compared with healthy controls. The obtained results of basophil activation in microfluidic chip were comparable with flow cytometry analysis.

The microfluidic device is coated with specific antibodies to CD203c to isolate basophils. Experimentally, the cell-capture efficiency drops with increased shear stress. This observation suggests that when target cells come into contact with the surface, cell–substrate adhesion is started. The sudden drop of cells captured at a higher flow rate indicates less time for antibody–cell contact. Hence, there seems to be a critical balance between specific cell capture and shear-induced cell loss. Furthermore, we confirm that a low number of basophils was captured when blood flowed through an unmodified chip, whereas the total number of leukocytes in the control chip compared with the CD203c-positive

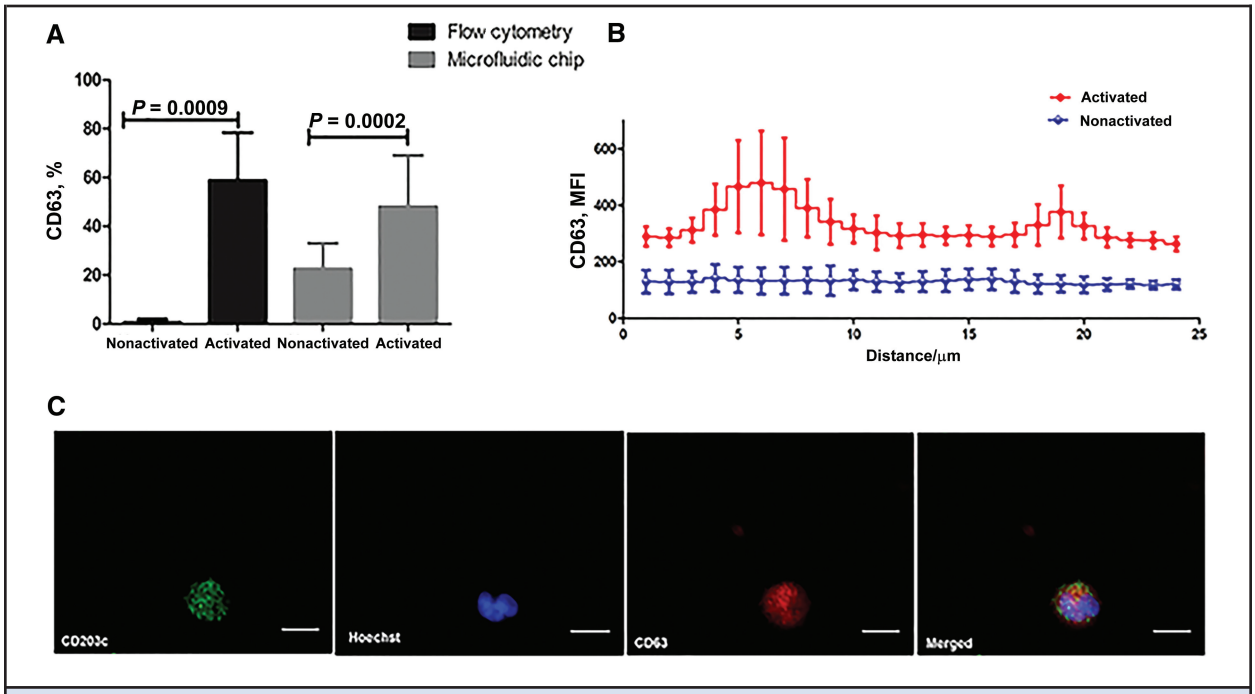

Fig. 5. Basophil expression of CD63 in microfluidic chip.
(A) CD63 expression (CD63%) of activated and nonactivated basophils in microfluidic chip parallel to flow cytometry analysis. Scatter plots representing the range with whiskers and a line as the median (n = 8). A *P* value of <0.05 was considered significant. (B), Histograms show CD63 MFI in activated captured basophils (red line) compared with nonactivated basophils (blue line). (C), The fluorescent staining of activated basophil captured in microfluidic chip. Green signal represents CD203c Alexa-488; blue signal represents nucleus staining (Hoechst stain); and the red signal represents CD63 Alexa-647. Scale bar: 10 μm.

chip did not differ significantly. This indicates that the majority of contaminating cells are nonspecifically bound, demonstrating that the antibody is specific to the basophil cells. For a flow cytometry-based basophil activation test for allergy diagnosis, the sufficient number of basophils gated from the sample to quantify CD63 expression is 200 basophils (*30*), and the microfluidic devices are capable of capturing more than this number without further optimization. The nonspecific binding of other leukocytes can be further reduced by optimizing the device geometry and flow conditions. Among the subpopulation, monocytes can express a low level of FcεRI and CD63. However, the activation mechanism of the FcεRI pathway in monocytes is different and requires a high concentration of stimuli and longer incubation time compared with cross-link FcεRI in basophils (*31*). Furthermore, because captured basophils are stained specifically with CD203c, this gives assurance to exclude other CD63 signal than CD203c-positive cells.

The CD203c MFI in captured activated basophils is significantly higher than in the nonactivated basophils. CD203c is a glycosylated type II transmembrane molecule, which is expressed constitutively on basophils. Moreover, the expression intensity of the CD203c is low to intermediate on resting basophils but becomes upregulated on activation. Together, the results demonstrate that the overexpression of CD203c after on-chip activation could be used as an activation marker on the microfluidic device.

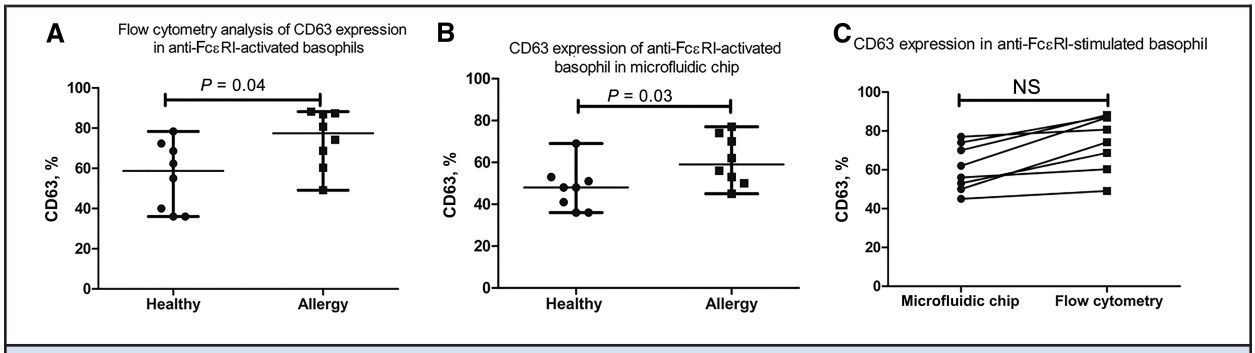

Fig. 6. CD63 expression in activated basophils comparing allergy patients with healthy controls. (A), CD63% expression of anti-FcεRI-activated captured basophils in microfluidic chip in allergy patients compared with healthy controls. Scatter plots representing the range with whiskers and a line as the median. (B), Flow cytometric analysis of CD63 expression of anti-FcεRI-activated basophils in allergy patients compared with healthy controls. Scatter plots representing the range with whiskers and a line as the median. (C), Nonsignificant difference in CD63 expression in activated basophils compared with microfluidic chip analysis and flow cytometry in allergy patients. A *P* value of <0.05 was considered significant.

The high negative background of CD63 expression in captured basophils has been previously discussed (*31*). There are several causes likely to be responsible for a high basal value in vitro, particularly pyrogens and endotoxins that could contaminate the materials used in the technique, such as plastic tubes or syringes. Therefore, it is important to work in a sterile environment (*31*) and redesign a chip with the possibility of minimizing the assay process time that might reduce the negative background. The mechanical stress formed during the cell-capturing process in the chip may prime the spontaneous activation of captured basophils and induce degranulation (*32*). However, we found that the CD63 MFI of anti-FcεRI-activated basophils at the single-cell level is significantly higher compared with nonactivated basophils. The CD63 MFI ratio can be potentially used to measure CD63 level in activated basophils (*33*). Furthermore, analysis of CD63 expression on the basophil cell surface has been shown to correlate to degranulation owing to activation of basophils by allergens (*34*). Our data show that the CD63 expression using microfluidic chip did not significantly differ compared with flow cytometry analysis. This indicates that the miBAT is sensitive enough to detect basophil activation.

In the future, we will improve the microfluidic method to enable testing of allergen activation using blood from a cohort of allergy patients. This will ensure sufficient statistical power of the miBAT. We also aim to develop automated sample processing and analysis to facilitate point-of-care testing at clinical settings.

CONCLUSION

We have developed a device capable of isolating CD203c-positive cells directly from whole blood for on-chip activation of the cells for allergy diagnosis. We have in this study demonstrated the ability to activate and detect the level of basophil activation assessed by CD63 expression. This technique provides a new and potentially useful method for measurement of basophil activation level, which could facilitate diagnosis and monitoring of allergic responses at the point of care.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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