Immune modulation by helminths and the impact on the development of type 2 diabetes

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A FIELD-APPLICABLE METHOD FOR FLOW CYTOMETRIC ANALYSIS OF GRANULOCYTE ACTIVATION: CRYOPRESERVATION OF FIXED GRANULOCYTES

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Cytometry Part A (2018)

ABSTRACT

Upon activation granulocytes upregulate several adhesion molecules (CD11b) and granule proteins (CD35, CD66b) and shed surface L-selectin (CD62L). These changes in expression, as assessed by flow cytometry, can be used as markers for activation. Whereas these markers are usually studied in fresh blood samples, a new method is required when samples are collected at a field site with no direct access to a flow cytometer. Therefore, we developed and tested a field-applicable method in which fixed leukocytes were cryopreserved. Using this method, the intensity of granulocyte activation markers was compared to samples that were either stained fresh, or fixed prior to staining but not cryopreserved. In addition, the response to an in vitro stimulation with fMLF was determined. While we observed differences in marker intensities when comparing fresh and fixed granulocytes, similar intensities were found between fixed cells that had been cryopreserved and fixed cells that did not undergo cryopreservation. Although fixation using FACS lysing solution might lead to membrane permeabilization, activation markers, and the responsiveness to fMLF or eotaxin could still be clearly measured. This method will, therefore, enable future studies of granulocyte activation in settings with limited resources and will allow simultaneous analysis of samples collected at different time points.

INTRODUCTION

Granulocytes form a class of innate effector cells and consist of eosinophils, neutrophils, and basophils, all characterized by the presence of lobulated nuclei and secretory granules in their cytoplasm. Upon inflammation, granulocytes are recruited from the circulation to the site of tissue injury or infection, where they become activated and exert their effector functions. To adhere to the endothelium, granulocytes express L-selectin (CD62L), and several selectin ligands which mediate "rolling" along the vessel endothelium (1, 2). This is followed by firm adhesion to and transmigration through endothelial cells, which is mainly mediated by the integrins Mac1 (CD11b/CD18) and VLA4 (CD49d/CD29, only expressed by eosinophils and basophils) (1, 2).

In addition to adhesion molecules, surface expression of proteins normally residing inside granule membranes can be used as markers for degranulation and, therefore, activation (3). Relevant are complement receptor 1 (CD35), mediating binding and phagocytosis of C3b coated particles and immune complexes, present in secretory vesicles, and CEACAM-8 (CD66b) which is present in specific granules (3).

Whereas neutrophils demonstrate a rapid upregulation of CD11b, CD35 and CD66b after in vitro activation with inflammatory agonists such as *N*-Formyl-Met-Leu-Phe (fMLF), the level of CD62L reduces as a result of stimulation-induced shedding (4, 5). Similarly, eosinophils respond to the chemokine eotaxin (amongst others) by upregulating CD11b and shedding their CD62L (6, 7). Therefore, quantification of these markers by flow cytometry can be used as read-out for the activation state and responsiveness of the cells for inflammatory mediators (4, 5, 8-14).

To study the expression of activation markers on granulocytes by flow cytometry, the use of lysed whole blood is preferred over cell isolation techniques as the latter could lead to nonspecific activation (15). In addition, purification procedures often require more time and a larger volume of blood compared to using whole blood. Therefore, total leukocytes are usually analysed by multicolour flow cytometry after staining whole blood with fluorescently-labelled antibodies followed by multiple gating (4, 5, 16). Whereas this method is suitable for laboratories with direct access to flow cytometers, circumstances are different when blood is collected at a field site with only basic laboratory infrastructure. Hence, a novel method is required in which samples can be collected in a setting with limited resources and stored for long time periods until analysis in a fully equipped laboratory.

Fixation methods are often used to preserve cells and studies have successfully demonstrated the applicability of granulocyte fixation prior to staining (8, 10, 17). However, fixation can modify cell morphology as measured by forward scatter (FSC) and side scatter (SSC) of leukocytes (8, 17-19). Fixation may also affect granulocyte cell membrane permeability (18-20) and can affect antigen epitopes and, thereby, their interaction with the monoclonal antibodies (9). This requires careful evaluation of each antigen-antibody combination.

As the cryopreservation of freshly isolated granulocytes has been proven difficult because of high susceptibility to damage during the cryopreservation method (21), cryopreservation of fixed lysed whole blood samples might be a suitable method for long-term storage. This would allow sample collection at the field site requiring only pipettes, a centrifuge and a -80°C freezer, followed by transport to a central laboratory for flow cytometric analysis. Moreover, long-term storage allows researchers to analyse samples collected at different time points simultaneously.

Nemes et al. previously provided proof-of-principle that cryopreservation leads to accurate quantification of cell subsets (granulocytes, lymphocytes, monocytes, T cells and B cells) by flow cytometry in fixed whole blood samples (17). Our study tested the hypothesis that also granulocyte activation can be determined in such samples by comparing the expression of granulocyte activation markers on samples that were either fresh (no fixation), fixed prior to staining, or fixed and cryopreserved prior to staining. In addition, we set out experiments to measure the response of granulocytes to an in vitro stimulation with fMLF (neutrophils or basophils) or eotaxin (CCL11; eosinophils) in cells fixed and cryopreserved after activation.

The applicability of cryopreservation of fixed cells was subsequently tested in a controlled laboratory model of acute inflammation (22) and in a field study in Flores, Indonesia (23). We now show that cryopreservation of fixed total leukocyte populations allows the determination of activated granulocytes, either activated by chemokines in vitro or by inflammation in vivo.

MATERIALS & METHODS

Sample preparation

Venous blood of three healthy volunteers was collected in sodium heparin vacutainers (BD Biosciences, Franklin Lakes, NJ, USA) and handled within 30 minutes. Different methods were compared, depicted in Figure 1, and for each method 2 polystyrene round-bottom tubes containing 200 µL of blood per donor were pre-incubated for 5 min in a 37°C waterbath, followed by a 5 min-stimulation at 37°C with fMLF (10^{-5} M; Sigma, Saint Louis, MO, USA) or left unstimulated. Subsequently, samples were subjected to the following treatments: (1) Erythrocyte lysis using lysing buffer consisting of 155 mM NH_4Cl and 10 mM Na_2EDTA, with a pH of 7.0 and osmolarity of 298-305. Cells were transferred to ice and to each sample 900 µL lysing buffer was added. Cells were centrifuged at 1600 rpm for 5 min and washed with first 500 µL lysis buffer, followed by washing and resuspension in FACS buffer (PBS complemented with 0.5% BSA and 2 mM EDTA) and directly stained. (2) Erythrocyte lysis and leukocyte fixation using FACS lysing solution (BD Biosciences, #349202) for 15 min at room temperature. Following the recommended procedures, the FACS lysing solution (stock solution 10x) was diluted, and 4 mL of the diluted reagent was used for each whole blood sample. After lysis the cells were centrifuged, washed with RPMI 1640 containing 10% heat-inactivated foetal calf serum (FCS), centrifuged again and

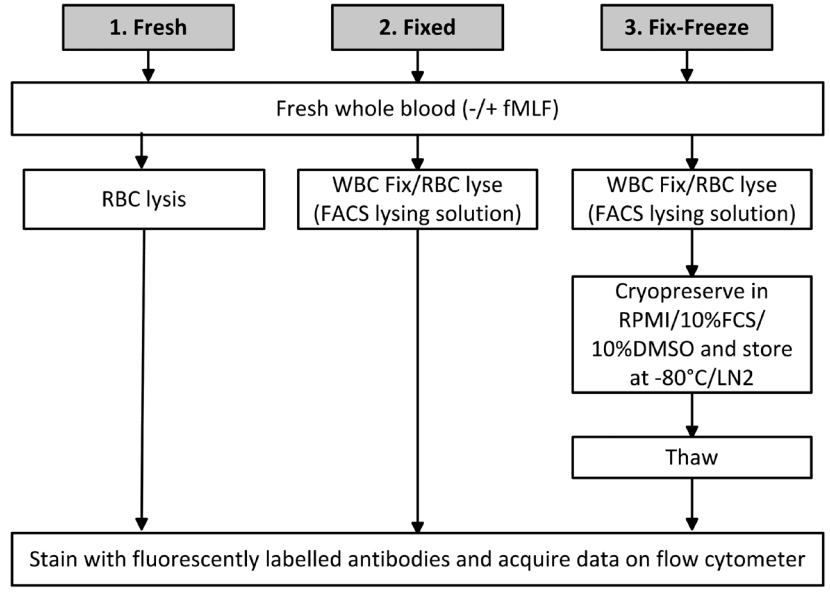

Figure 1. Experiment overview.

finally resuspended in FACS buffer and stored (<8 hrs) at 4°C until staining. (3) Erythrocyte lysis and leukocyte fixation using FACS lysing solution as described above (Treatment 2). Following lysis, cells were washed with 10% FCS/RPMI and resuspended in 10% FCS/10% dimethyl sulfoxide (DMSO)/RPMI. Cryovials containing the cell suspension were placed at -80°C. The cryopreserved fixed cells were thawed on the bench and subsequently transferred to 5 ml polystyrene round-bottom tubes containing 1 mL 10% FCS/RPMI. After centrifugation at 1600 RPM for 5 min, cells were resuspended in FACS buffer and stored (<2 hrs) at 4°C until staining. In the results section, Treatments 1, 2 and 3 will be referred to as "Fresh", "Fixed" and "Fix & Freeze", respectively.

Cryopreservation of fixed leukocytes (Treatment 3), was applied in a clinical trial conducted in a rural area at Flores island in Indonesia to study the effect of soil-transmitted helminth infections on granulocyte activation. This SUGARSPIN trial was approved by the ethics committee of Faculty of Medicine, Universitas Indonesia (FKUI), filed by the ethics committee of Leiden University Medical Center (LUMC), and registered as a clinical trial (http://www.isrctn.com/ISRCTN75636394). The study protocol was published previously (23). Blood samples were collected after obtaining written consent and processed according to Treatment 3, with the only difference that cells were stored in liquid nitrogen after placement at -80°C for a minimum of 4 hours. In addition to the stimulation with fMLF described above, whole blood was stimulated for 5 min at 37°C with eotaxin (CCL11; 10^{-7} M, R&D systems, Abingdon, UK) to study in vitro eosinophil activation.

Neutrophil subset identification after LPS challenge

To assess whether neutrophil activation can also be detected after using the method of fixation and cryopreservation following in vivo stimulation, cells from a large endotoxin trial (NCT02629874 at www.clinicaltrials.gov) were used. In short, healthy male volunteers were enrolled after screening and were prehydrated. U.S. Reference *E. coli* endotoxin (Lot Ec-5; Centre for Biologic Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD, USA) was used in this study. Endotoxin was reconstituted in 5 mL saline and injected as a single intravenous bolus (2 ng/kg) during 1 min at t = 0. The study protocol was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and complies with the Declaration of Helsinki and Good Clinical Practice guidelines. Subjects gave written informed consent.

At t = 180 min, blood samples, anticoagulated with sodium heparin, were taken from the arterial catheter. The blood samples were processed according to Treatments 1 and 3 described above. For Treatment 3, cells were first stored at -30°C for 8 days for operational reasons (no -80°C freezer at sampling site), before they were transferred to -80°C.

Flow cytometric analysis

Irrespective of the method used, cells were counted by microscopy after erythrocyte lysis and 500.000 leukocytes were transferred to a 96-well V-bottom microplate and incubated for 30 min at 4°C with anti-CD35-FITC (E11, Biolegend, San Diego, USA), anti-CD66b-PerCP/Cy5.5 (G10F5, Biolegend), anti-CD193-PE (5e, Biolegend), anti-CD16-PE/CF594 (3G8, BD Biosciences), anti-CD69-PE/Cy5 (FN50, Biolegend), anti-CD3/CD19/CD20/CD56-APC (UCHT1, HIB19, 2H7, 5.1H11, Biolegend), anti-CD11b-APC/eF780 (ICRF44, eBioscience Inc., San Diego, USA), anti-CD203c-BV421 (NP4D6, Biolegend), anti-CD14-BV510 (M5E2, Biolegend) and anti-CD62L-BV605 (DREG-56, BD Biosciences). Cells collected after LPS challenge were stained with anti-CD16-Pacific Orange (3G8, Beckman Coulter) and CD62L-BV650 (DREG-56, Biolegend). Compensation beads (BD Biosciences) were stained with each antibody separately and run before acquisition to calculate the compensation matrix using BD Facs Diva flow cytometry software.

As we aimed to compare three methods, all samples were measured on the same day and stained with the same antibody mix, with exception of the samples that were collected in the endotoxin clinical trial or as part of the SUGARSPIN study. Cells were measured on a LSR Fortessa flow cytometer (BD Biosciences), equipped with 405, 488, 561 and 640 lasers. Data were analysed with FlowJo software (Treestar Inc., Ashland, OR, USA) and median fluorescence intensity (MFI) data are displayed. Representative gating schemes are shown in Supplementary Figure S1. Although neutrophils and eosinophils clearly differ in their FSC/SSC properties, we used the expression of CD193 (in fresh samples) or the autofluorescent signal (in fixed samples) to gate eosinophils. Neutrophils were gated based on their FSC/SSC properties and subsequently separated from monocytes based on their $CD14^{-}/CD11b^{+}$ expression. Basophils were gated based on their expression of CD193 and CD203c and lack of the lineage markers CD3, CD19, CD20 and CD56.

Statistical analysis

Data analysis was performed using GraphPad Prism version 7 for Windows (Graphpad Software, San Diego, CA, USA) and this software was also used to make graphs. Paired t test was used to assess the effect of fMLF stimulation on the expression of activation markers in neutrophils and basophils. The effect of stimulation on markers expressed by neutrophils, eosinophils and basophils from samples collected in a field study was tested using Wilcoxon matched-pairs signed rank test. P values ≤ 0.05 were considered statistically significant.

RESULTS

Fixation leads to changes in FSC/SSC profiles

After fixation, granulocytes displayed different forward scatter/ side scatter (FSC/SSC) profiles compared to fresh cells suggesting a change in morphology (Figure 2). Eosinophils showed a shift in FSC and became more autofluorescent after fixation (Supplementary Figure S2). No increase in autofluorescence was observed for neutrophils (data not shown). After fixation, neutrophils exhibited a lower SSC signal, resulting in an overlap between neutrophils and monocytes. The amount of overlap increased after freeze-thawing, and it was, therefore, important to add a monocyte marker (e.g. CD14) and/or a specific neutrophil marker (e.g. CD66b) to the panel to be able to discriminate between these cell types. Except for neutrophils, fixed leukocytes that had been cryopreserved displayed similar FSC-SSC properties compared to fixed cells that did not undergo cryopreservation.

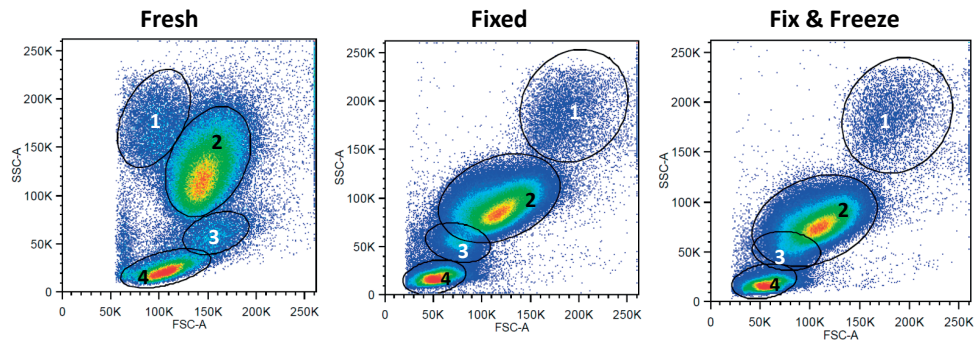

Figure 2. FSC/SSC properties of granulocytes. Numbers indicate different cell populations: (1) eosinophils, (2) neutrophils, (3) monocytes and (4) lymphocytes. After fixation, eosinophils change in FSC/SSC and monocytes and neutrophils show overlap in FSC/SSC properties. This overlap is further amplified after freeze-thawing the cells. Basophils overlap with lymphocytes in FSC/SSC properties.

Neutrophils

Cellular activation and responsiveness to fMLF can be measured by activation markers after fixation and cryopreservation

We observed changes in marker intensities when comparing fresh and fixed unstimulated neutrophils (Figure 3, Table 1). The measured intensities of CD11b, CD35 and CD66b were elevated after fixation when compared to fresh cells, whereas the detected intensity of CD62L on neutrophils was lower after fixation compared to fresh cells. These changes were consistent in all three donors. No major differences in the marker intensities were observed when fixed neutrophils that had been cryopreserved were compared to fixed cells that did not undergo cryopreservation (Figure 3), indicating that the observed differences with fresh cells are a consequence of fixation, and are not caused by the process of freeze-thawing.

To assess whether it is possible to detect activation of neutrophils after fixation and cryopreservation, we measured the levels of activation markers in samples that were incubated with and without fMLF. Despite the changes in marker intensities that were described above, fMLF activation could still further increase the expression of measured activation markers in fixed and cryopreserved neutrophils (Figure 3, Table 1). The detected responsiveness of CD35 and CD11b to fMLF was similar in fresh and fixed neutrophils, irrespective of freezing. Regarding CD66b, we observed an increased expression after stimulation with fMLF in fresh neutrophils as well as fixed cells. However, in fixed cells, the increase in CD66b expression was rather weak compared to fresh cells, indicating that CD66b might not be suitable for studying functional capacity of neutrophils in terms of responding to a stimulus when this field applicable method is used. CD62L is shed from the membrane upon activation, and despite the overall lower intensity of CD62L in fixed neutrophils, we could still observe this response after activation with fMLF.

Three neutrophil subsets can be identified based on CD16 and CD62L expression after fixation and cryopreservation

The experiments described above relied on the in vitro activation of leukocytes by fMLF. We next studied leukocyte subsets that have been described in fresh cells following

Table 1. Expression of CD11b, CD35, CD66b and CD62L on neutrophils and the response to fMLF.

Neutrophils	Fresh Mean MFI (SD)		Fixed Mean MFI (SD)		Fix & Freeze Mean MFI (SD)	
	Medium	fMLF	Medium	fMLF	Medium	fMLF
CD11b	164 (20)	857 (124)	1175 (101)	1758 (222)	1525 (132)	2154 (186)
CD35	788 (92)	4054 (921)	1113 (160)	3729 (832)	1399 (99)	3981 (845)
CD66b	337 (25)	909 (113)	518 (62)	583 (18)	590 (74)	720 (17)
CD62L	5304 (753)	261 (49)	1216 (108)	169 (8)	1118 (41)	156 (8)

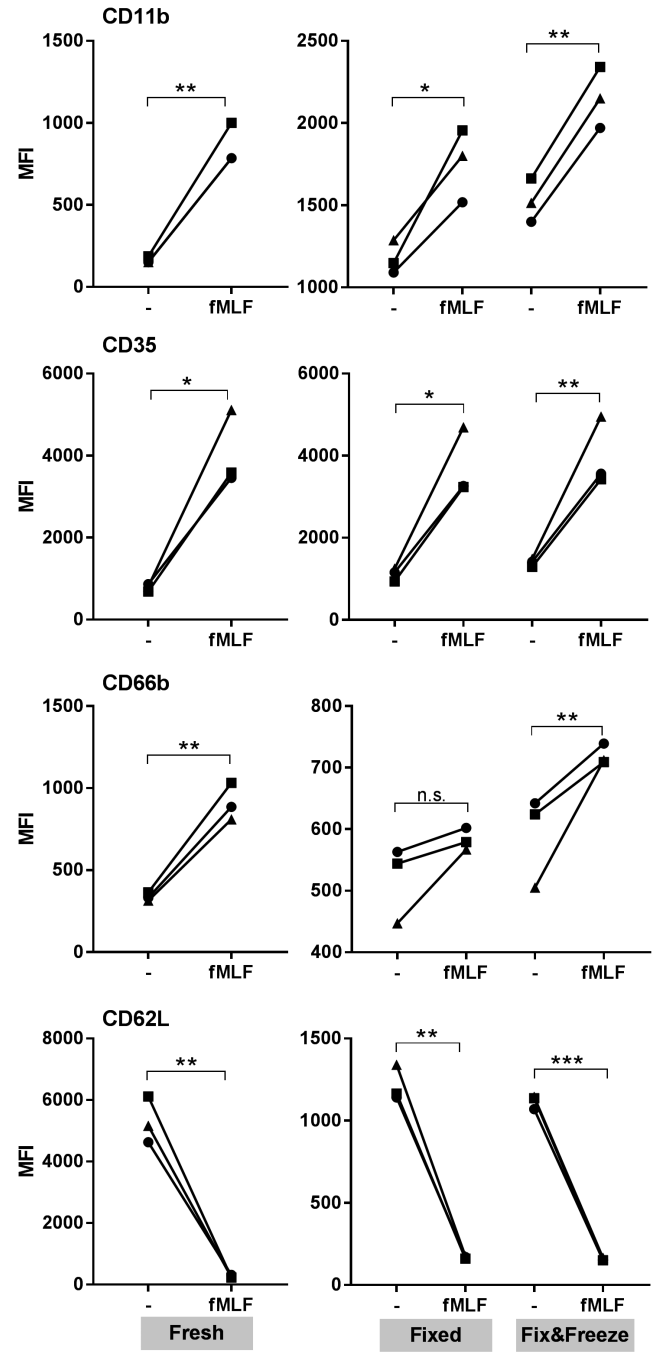

Figure 3. Expression of CD11b, CD35, CD66b and CD62L on neutrophils and the response to fMLF. Median fluorescent intensity (MFI) of markers on neutrophils left unstimulated and stimulated with fMLF. Neutrophils were stained either fresh (Fresh), after fixation (Fixed) or after being fixed, cryopreserved and thawed (Fix & Freeze). Symbols (circle, triangle, square) represent three different donors. *indicates analysis with paired *t* test. *, $P \leq 0.05$. **, $P \leq 0.01$. ***, $P \leq 0.001$. n.s., not significant.

in vivo stimulation by a systemic pro-inflammatory stimulus, LPS. Thus, since CD62L intensities were found to be decreased after fixation, we assessed whether we would still be able to identify the three different neutrophil subsets that were previously described on fresh cells (22). Although both CD16 and CD62L intensities were lower after fixation and cryopreservation, we were able to identify the three different neutrophil subsets (1- $CD16^{bright}CD62L^{bright}$; 2- $CD16^{dim}CD62L^{bright}$; 3- $CD16^{bright}CD62L^{dim}$) based on expression of these two markers (Supplementary Figure S3).

Eosinophils and Basophils

The intensities of all eosinophil markers studied (CD193, CD66b, CD35, CD11b, CD62L and CD69) were similar between fixed eosinophils that had been cryopreserved and fixed cells that did not undergo cryopreservation (Figure 4). However, similar to the situation with neutrophils, differences were observed in the intensities of activation markers for eosinophils when comparing fresh and fixed cells. The measured intensities of CD35 and CD66b were slightly higher after fixation, while the intensity of CD11b was strongly increased and CD62L detection was lower in fixed compared to fresh cells.

Basophils also respond to fMLF (24) and the intensity of CD11b, CD35 and CD203c was increased after stimulation (Supplementary Figure S4, Table S1). Like the expression of these markers in unstimulated cells, the responsiveness to fMLF was similar between fresh, fixed and cryopreserved cells.

Field study application

The aim of this study was to set up a method to assess granulocyte activation and responsiveness in a field study with limited laboratory equipment. After testing the method to fix and cryopreserve leukocytes for flow cytometric analysis, this method was applied in a clinical trial in a rural area in Indonesia (23). Blood samples from 300 subjects were processed according to the method described and stored in liquid nitrogen for up to two years until analysis. Figures 5 and 6 and Supplementary Figure S5 show representative samples collected during this study. Even after long-term storage we could measure the expression of activation markers on neutrophils (Figure 5A), eosinophils (Figure 6A) and basophils (Supplementary Figure S5A). Moreover, the response of neutrophils and basophils to fMLF evoked in samples in the field was clearly present (Figure 5B, Supplementary Figure S5B, Table S2). To study the responsiveness of eosinophils, we stimulated whole blood with eotaxin and observed an increased intensity of CD35 and CD11b when compared to unstimulated cells (Figure 6B, Table S2). Although not compared to fresh cells, this shows that the activation effect in eosinophils can be measured after fixation and cryopreservation.

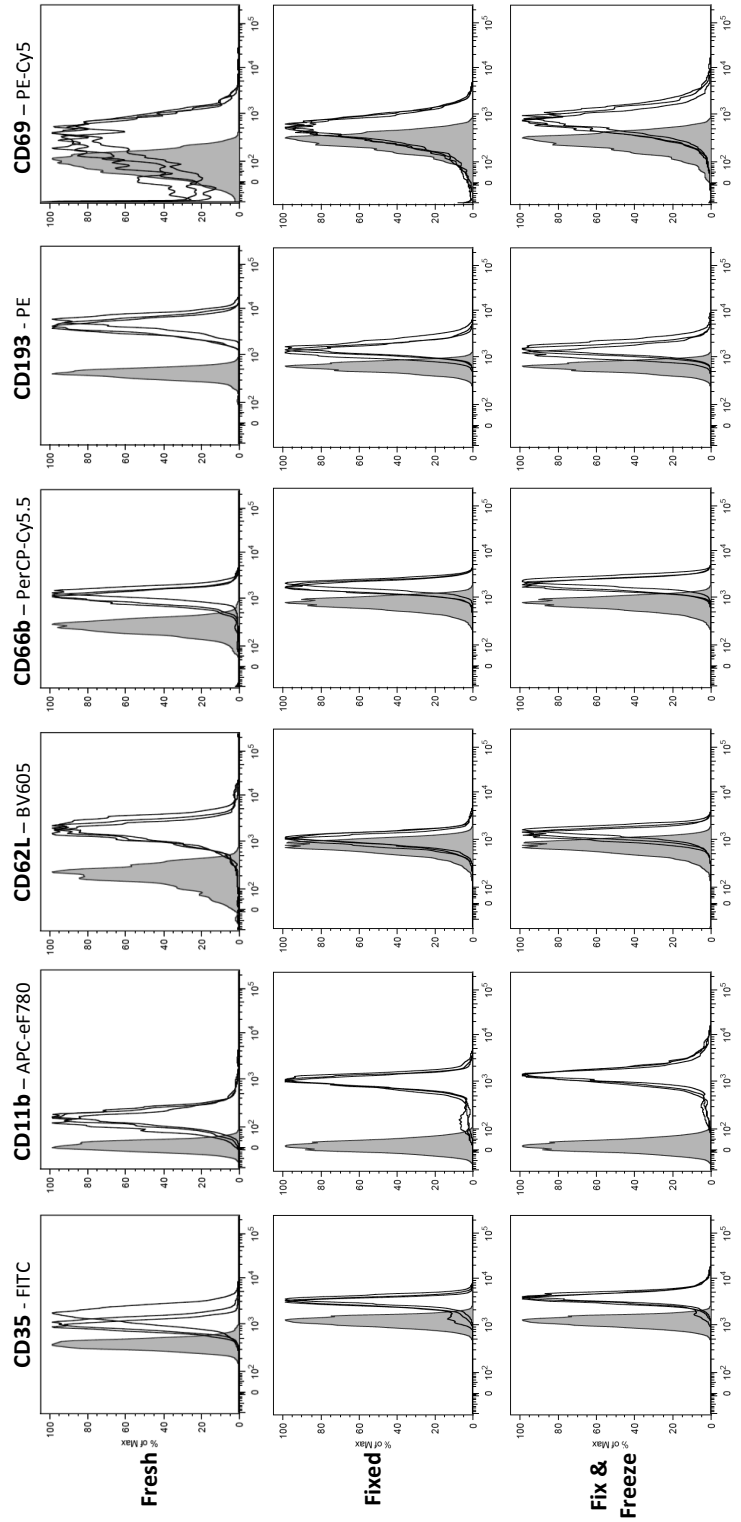

Figure 4. Expression of CD35, CD11b, CD62L, CD66b, CD193 and CD69 on eosinophils. Grey histograms represent unstained eosinophils while the black lines represent three different donors. Cells were stained either fresh (Fresh), after fixation (Fixed) or after being fixed, cryopreserved and thawed (Fix & Freeze).

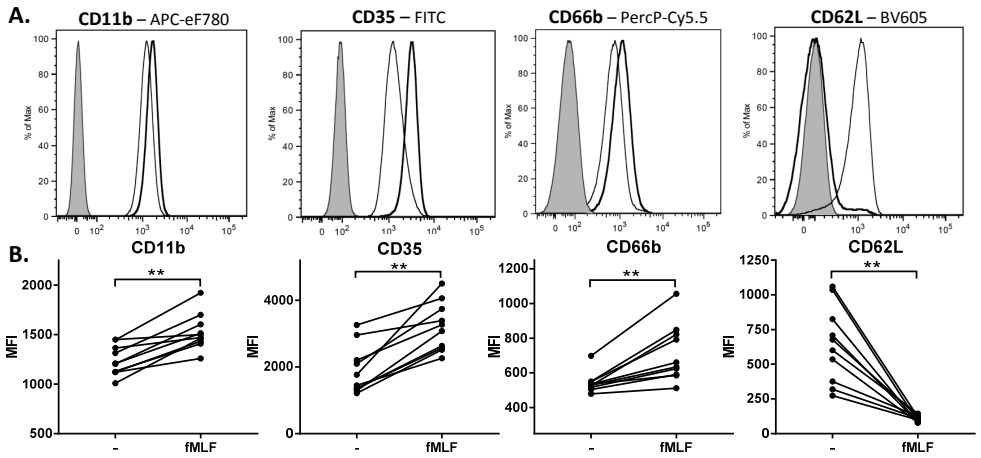

Figure 5. Expression of CD11b, CD35, CD66b and CD62L on neutrophils collected in a field study. Samples were cryopreserved for 2 years in liquid nitrogen. (*A*) Histograms of a representative sample, filled lines display unstained neutrophils, the thin black lines unstimulated cells and the thick black lines fMLF-stimulated cells. (*B*) Median fluorescent intensity (MFI) of markers in neutrophils from 10 representative donors left unstimulated and stimulated with fMLF. Means and standard deviations are shown in Supplementary Table S2. *indicates analysis with Wilcoxon matched-pairs signed rank test. **, $P \leq 0.01$.

DISCUSSION

Here, we show that the activation status of neutrophils, eosinophils, and basophils and their responsiveness to stimuli such as fMLF and eotaxin, can be studied in whole-blood samples that were lysed, fixed and cryopreserved. Previously, Nemes et al. showed that cryopreservation of fixed leukocytes provides for an accurate quantification of cell subsets based on the expression of lineage-specific markers (CD66b, CD14, CD3, CD19, CD16), as analysed by flow cytometry (17). However, they had not tested whether the method was also suitable for assessing the activation status of granulocytes. We show that cryopreservation of granulocytes allows detection of activation of granulocytes in vitro in response to fMLF and eotaxin, as well as activation in vivo during acute inflammation evoked by experimental endotoxemia in healthy volunteers. It will now be possible to measure differences in innate immune responses at field sites where no flow cytometers are available for direct analysis.

Cryopreservation of fixed granulocytes induces some differences in cellular characteristics that preclude direct comparison with analysis of fresh cells. For instance, the differences detected in FSC/SSC profiles of granulocytes and marker intensities between cryopreserved and fresh cells were mainly caused by fixation. The increased intensity of CD11b, CD35 and CD66b in fixed neutrophils might be a consequence of fixation-induced membrane permeabilization, potentially leading to intracellular staining in the granules (19, 20). This is particularly relevant for markers normally residing in the granular

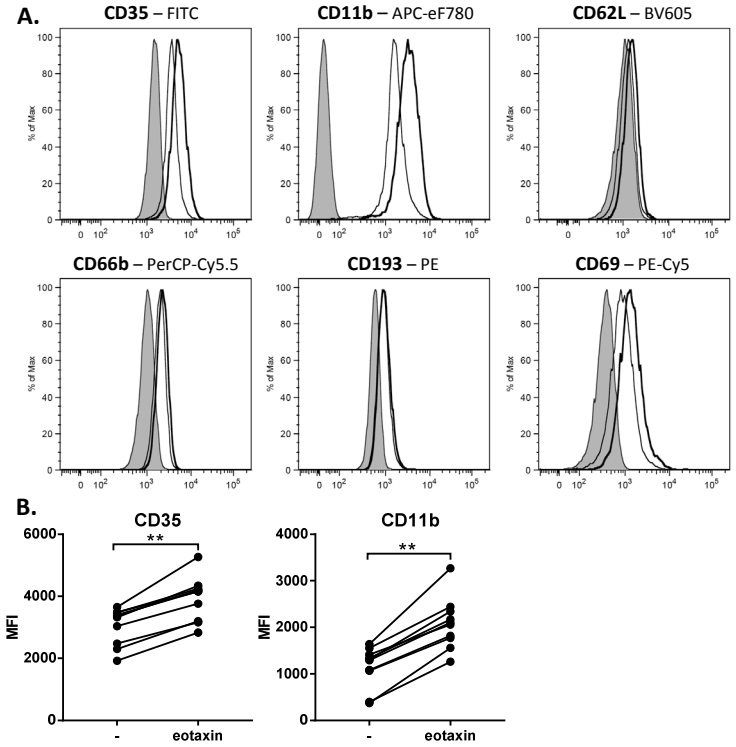

Figure 6. Expression of CD35, CD11b, CD62L, CD66b, CD193 and CD69 on eosinophils collected in a field study. Samples were cryopreserved for 2 years in liquid nitrogen. (*A*) Histograms of a representative sample, filled lines represent unstained eosinophils, the thin black lines unstimulated cells and the thick black lines eotaxin-stimulated cells. (*B*) Median fluorescent intensity (MFI) of CD35 and CD11b in eosinophils from 10 donors left unstimulated and stimulated with eotaxin. Means and standard deviations are shown in Supplementary Table S2. *indicates analysis with Wilcoxon matched-pairs signed rank test. **, $P \leq 0.01$.

compartment, which become expressed upon degranulation. Only 5% of the total cellular content of the CD11b-CD18 (Mac-1) complexes in unstimulated neutrophils is expressed on the plasma membrane, whereas 95% colocalized with specific granules and secretory vesicles (25). Therefore, the increased intensity of CD11b in fixed versus fresh neutrophils might reflect some staining of the intracellular pool of CD11b molecules. Importantly, despite the putative membrane permeability caused by fixation, we could still observe a significant effect of fMLF in neutrophils (in particular for CD35, CD11b and CD62L) and basophils (in particular for CD203c, CD35 and CD11b), and of eotaxin in eosinophils (in particular for CD35 and CD11b). This activation effect was also clearly present in samples collected in the field which had been cryopreserved for up to two years.

Hamblin et al. observed no intracellular leukocyte staining of p8,14 (expressed in all circulating monocytes and neutrophils) nor DNA staining after fixation with 0.4%

formaldehyde (8). This suggests that different fixation methods might have different effects in terms of cell permeabilization. However, the use of FACS lysing solution is a quick and easy method and, therefore, suitable to be used in the field.

We also observed that eosinophils became more autofluorescent after fixation and it is, therefore, difficult to determine in eosinophils whether the differences in marker intensity between fresh and fixed cells are a result of cell membrane permeabilization, affected antigen epitopes, or increased autofluorescence. The effect of autofluorescence on the intensity of a certain marker depended on the fluorescent channel being used, as we observed no shift in the channel used to detect APC/eF780. CD193 (chemokine receptor CCR3 / eotaxin receptor) is a well-known marker for eosinophils and we observed a lower detection of CD193 in fixed compared to fresh eosinophils. Combined with an increased autofluorescent signal in the PE-channel, we recommend not to use CD193 as single eosinophil marker when analysing fixed, cryopreserved granulocytes. CD193 functioned well as a basophil marker, irrespective of fixation (26, 27). For similar reasons, we advise to use a CD62L antibody with a different fluorophore than BV605, or to increase the amount of antibody when staining fixed eosinophils to be able to detect CD62L.

In summary, these results demonstrate that fixation and cryopreservation of lysed whole blood samples can be applied at field sites with limited laboratory infrastructure. We showed that the activation status of granulocytes and responsiveness to fMLF or eotaxin could be measured after cryopreservation, allowing to measure changes in the innate immune response in patients. This will enable future studies of granulocyte activation in settings with limited resources and allows the parallel analysis of samples collected at different time points and at different sites.

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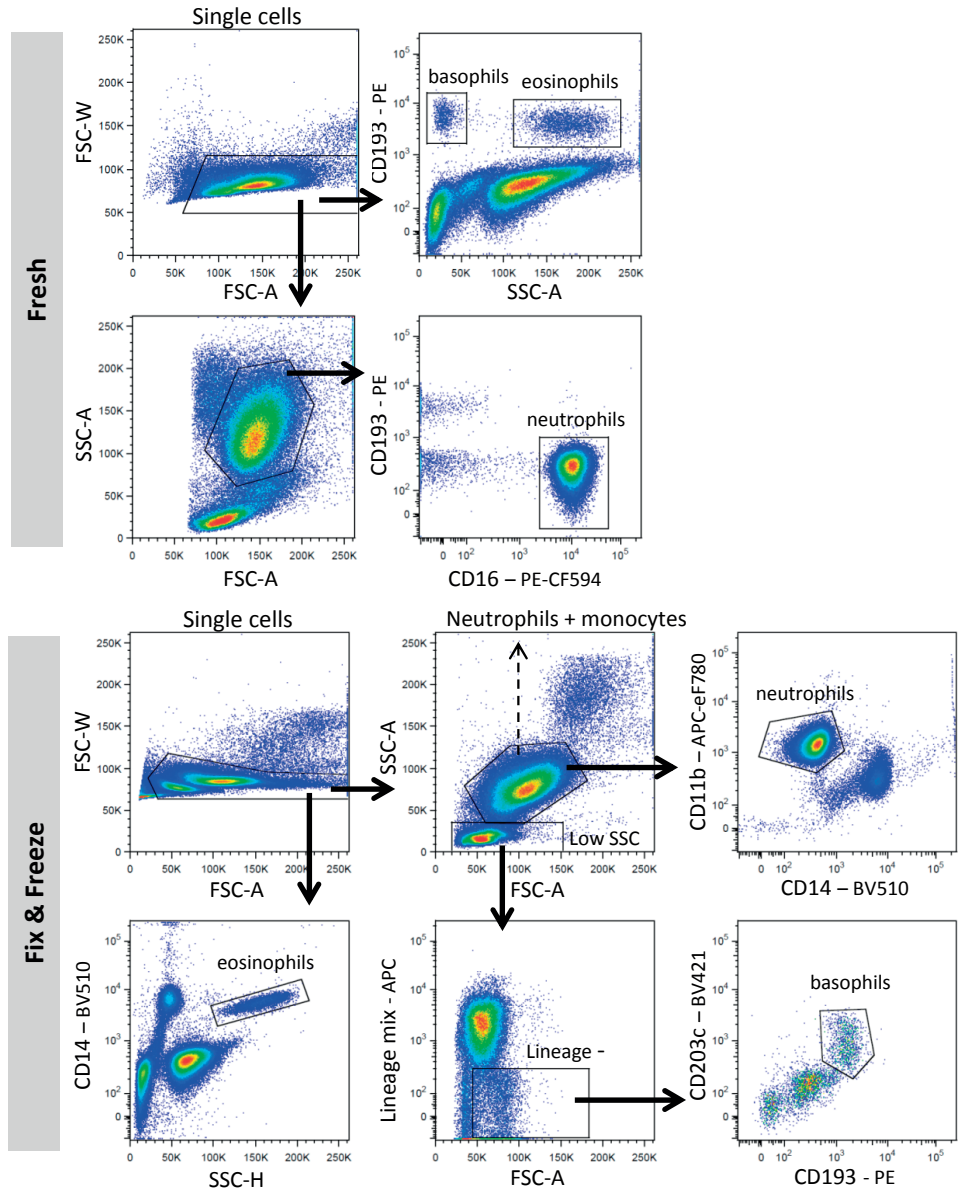
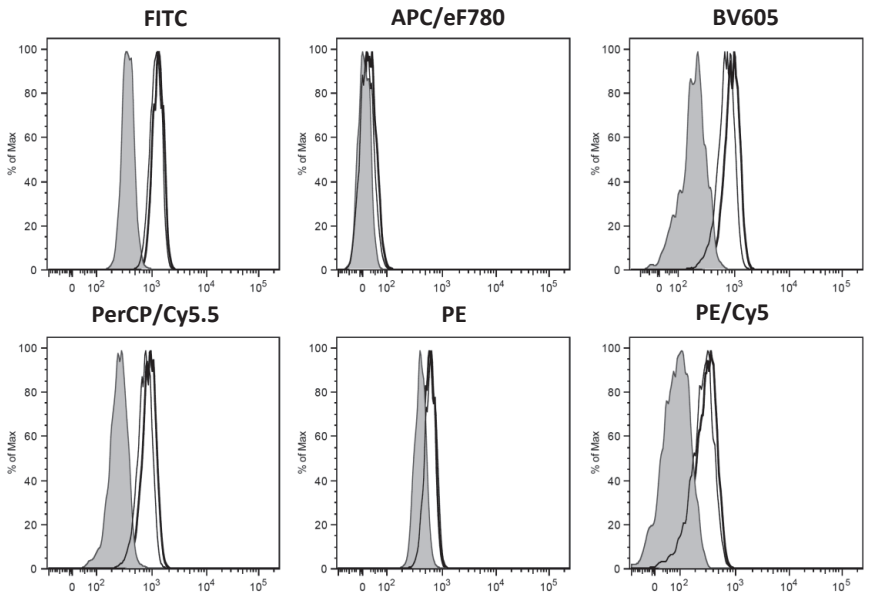
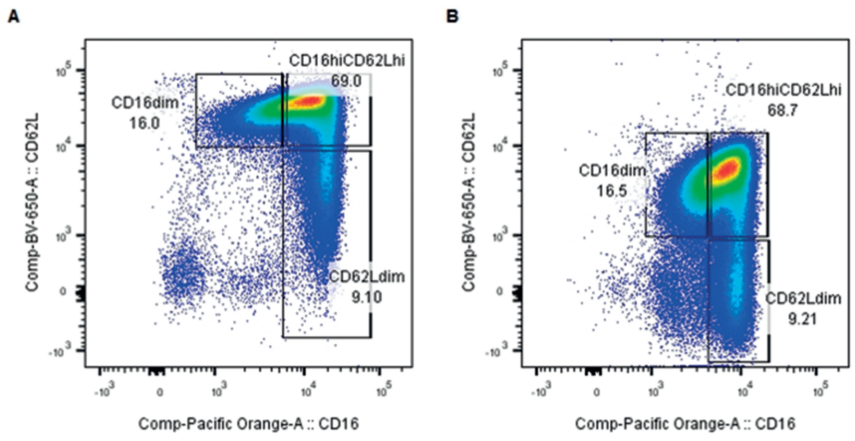
SUPPLEMENTAL DATA



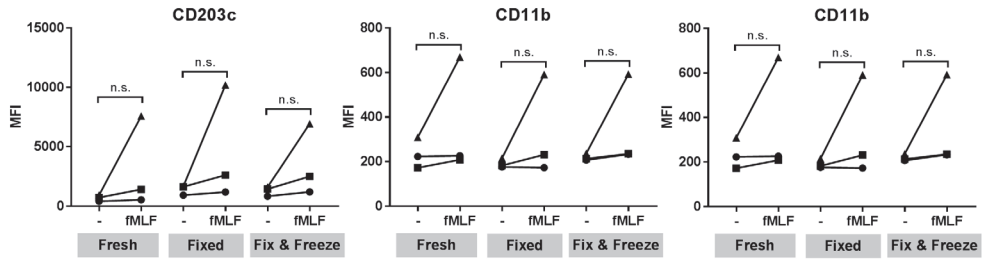
Supplementary Figure S1. Gating strategies. The gating strategy for the analysis of eosinophils, neutrophils and basophils in a representative fresh sample (upper panel) and fixed-cryopreserved sample (lower panel) is shown.



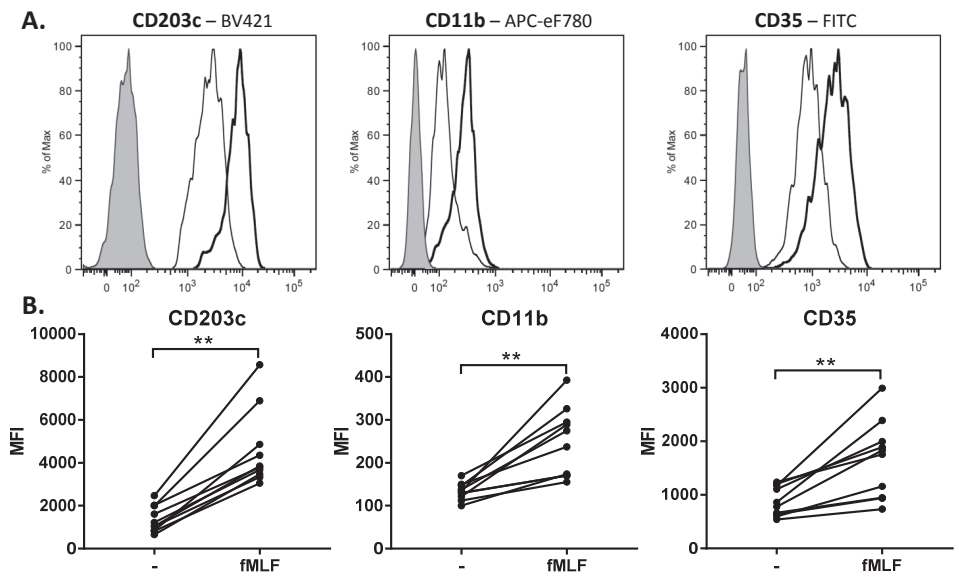
Supplementary Figure S2. Increased autofluorescence of eosinophils after fixation. The filled lines represent fresh, non-fixed, unstained eosinophils, while the thin black lines display fixed unstained cells that did not undergo cryopreservation and the thick black lines fixed, unstained cells after cryopreservation.



Supplementary Figure S3. Expression of CD203c, CD11b and CD35 on basophils and the response to fMLF. (*A*) Median fluorescent intensity (MFI) of markers on basophils left unstimulated and stimulated with fMLF. (*B*) The response of basophils to fMLF expressed as the difference in MFI between stimulated and unstimulated cells. The symbols (circle, triangle, square) represent three different donors. Means and standard deviations are shown in Supplementary Table S1. Data analysed with paired *t* test. n.s., not significant.



Supplementary Figure S4. CD16/CD62L expression of neutrophils that were activated in vivo in response to intravenous LPS infusion. Two extra neutrophil subsets (CD16^{dim}CD62L^{hi} and CD16^{hi}CD62L^{dim}) can be identified in both (*A*) fresh and (*B*) fixed and cryopreserved cells.



Supplementary Figure S5. Expression of CD203c, CD11b and CD35 on basophils collected in a field study. Samples were cryopreserved for 2 years in liquid nitrogen. (*A*) Histograms of a representative sample, filled lines display a fluorescence-minus-one (FMO) sample, the thin black line unstimulated cells and the thick black line fMLF-stimulated cells. (*B*) Median fluorescent intensity (MFI) of markers in basophils from 10 donors left unstimulated and stimulated with fMLF. Means and standard deviations are shown in Supplementary Table S2. *indicates analysis with Wilcoxon matched-pairs signed rank test. **, $P \leq 0.01$.

Supplementary table S1. Expression of CD203c, CD11b and CD35 on basophils and the response to fMLF.

	Fresh Mean MFI (SD)		Fixed Mean MFI (SD)		Fix & Freeze Mean MFI (SD)	
Basophils	Medium	fMLF	Medium	fMLF	Medium	fMLF
CD203c	682 (260)	3172 (3844)	1397 (417)	4661 (4840)	1288 (401)	3542 (3007)
CD11b	234 (69)	368 (261)	191 (21)	332 (226)	219 (15)	353 (207)
CD35	582 (148)	1361 (1122)	886 (157)	1618 (938)	888 (157)	1538 (779)

Supplementary table S2. Expression of activation markers on neutrophils, eosinophils and basophils from 10 representative donors collected in a field study presented as mean MFI and standard deviation.

Neutrophils	Medium	fMLF
CD11b	1237 (151)	1527 (181)
CD35	1895 (728)	3203 (735)
CD66b	543 (59)	713 (164)
CD62L	641 (278)	105 (23)
Eosinophils	**Medium**	**Eotaxin**
CD35	3044 (596)	3933 (714)
CD11b	1147 (438)	2079 (550)
Basophils	**Medium**	**fMLF**
CD203c	1374 (615)	4587 (1779)
CD11b	133 (20)	249 (80)
CD35	880 (275)	1663 (718)