



## Research paper

## Automated flow cytometry enables high performance point-of-care analysis of leukocyte phenotypes

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## ARTICLE INFO

## Keywords:

Phagocytes  
Inflammatory cells  
Flow cytometry  
Activation antibodies  
Fast analysis  
Point-of-care

## ABSTRACT

**Introduction:** Phagocytes such as granulocytes and monocytes are fundamental players in the innate immune system. Activation of these cells can be quantified by the measurement of activation marker expression using flow cytometry. Analysis of receptor expression on inflammatory cells facilitates the diagnosis of inflammatory diseases and can be used to determine the extent of inflammation. However, several major limitations of this analysis precludes application of inflammation monitoring in clinical practice. Fast and automated analysis would minimize *ex vivo* manipulation and allow reproducible processing. The aim of this study was to evaluate a fully automated “load & go” flow cytometer for analyzing activation of granulocytes and monocytes in a clinically applicable setting.

**Methods:** Blood samples were obtained from 10 anonymous and healthy volunteers between the age of 18 and 65 years. Granulocyte and monocyte activation was determined by the use of the markers CD35, CD11b and CD10 measured in the automated AQUIOS CL® “load & go” flow cytometer. This machine is able to pierce the tube caps, add antibodies, lyse and measure the sample within 20 min after vena puncture. Reproducibility tests were performed to test the stability of activation marker expression on phagocytes. The expression of activation markers was measured at different time points after blood drawing to analyze the effect of bench time on granulocyte and monocyte activation.

**Results:** The duplicate experiments demonstrate a high reproducibility of the measurements of the activation state of phagocytes. Healthy controls showed a very homogenous expression of activation markers at  $T = 0$  (immediately after vena puncture). Activation markers on neutrophils were already significantly increased after 1 h ( $T = 1$ ) depicted as means (95%CI) CD35:  $2.2 \times (1.5 \times -2.5 \times)$   $p = .028$ , CD11b:  $2.5 \times (1.7 \times -3.1 \times)$   $p = .023$ , CD10:  $2.5 \times (2.1 \times -2.7 \times)$   $p = .009$  and a further increase in activation markers was observed after 2 and 3 h. Monocytes also showed a increase in activation markers in 1 h (mean (95%CI) CD35:  $1.8 \times (1.3 \times -2.2 \times)$   $p = .058$ , CD11b:  $2.13 \times (1.6 \times -2.4 \times)$   $p = .025$ ) and also a further significant increase in 2 and 3 h was observed.

**Conclusion:** This study showed that bench time of one hour already leads to a significant upregulation and bigger variance in activation markers of granulocytes and monocytes. In addition, it is likely that automated flow cytometry reduces intra-assay variability in the analysis of activation markers on inflammatory cells. Therefore, we found that it is of utmost importance to perform immune activation analysis as fast as possible to prevent drawing wrong conclusions. Automated flow cytometry is able to reduce this analysis from 2 h to only 15–20 min without the need of dedicated personnel and in a point-of-care context. This now allows fast and automated inflammation monitoring in blood samples obtained from a variety of patient groups.

**Fund:** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Abbreviations:** fMLF, formyl-methionyl-leucyl-phenylalanine; MFI, median fluorescent intensity; HCs, Healthy controls; CI, Clearance interval; CR, Complement receptor

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<https://doi.org/10.1016/j.jim.2019.112646>

Received 27 May 2019; Received in revised form 9 August 2019; Accepted 12 August 2019

Available online 13 August 2019

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## 1. Introduction

Phagocytes such as neutrophils, monocytes and eosinophils are fundamental players of the innate immune system. They are easily activated by inflammatory stimuli and are typically the first cells to arrive at the site of infection (Kolaczowska and Kubes, 2013). For long it was thought that the phagocytes belong to a pool of cells with the elimination of pathogens as their main function (Kubes, 2018; Olingy et al., 2019). However, recently it has been recognized that these phagocytes also have other immune functions including tissue repair, tumor surveillance, modulating chronic inflammation and the ability to direct adaptive immune responses (Hellebrekers et al., 2018; Kolaczowska and Kubes, 2013; Kubes, 2018; Leliefeld et al., 2015). Phagocytes are involved in a range of disorders either associated with excessive cellular activation (e.g. systemic inflammatory response syndrome or chronic inflammatory diseases) or insufficient activation (chronic granulomatous disease or immune suppression after trauma) (Hellebrekers et al., 2018; Hesselink et al., 2019). Therefore, better understanding of *in vivo* activation of granulocytes and monocytes is crucial to get a better understanding of the etiology of these immune disorders.

Activation of granulocytes and monocytes can be quantified by measurement of the expression of activation markers (e.g. Mac1/CD11b, CR1/CD35, neutral endopeptidase/neprilysin/CD10) using flow cytometry (Arbra et al., 2017; Crockard et al., 1992; Eksioğlu-Demiralp et al., 2001; Mann and Chung, 2006; Martens et al., 1999). Analysis of receptor expression on inflammatory cells facilitates the diagnosis of inflammatory diseases and can be used to determine the extent of inflammation (Gernez et al., 2010; Hazeldine et al., 2017). However, several major limitations of this analysis precludes the broad application of flow cytometry for inflammation monitoring in clinical practice. These include the small window of opportunity for analysis because neutrophil receptors quickly change *in vivo*, the fact that inflammatory cells are easily activated by *ex vivo* manipulation and that the analysis is time-consuming and can only be performed by experienced laboratory personnel (Alvarez-Larrán et al., 2005; Cabrera et al., 2017; Hazeldine et al., 2017). Therefore, inflammation monitoring by flow cytometry is still mainly applied in research settings nowadays.

Recently, a fully automated flow cytometer, the AQUIOS CL® flow cytometer, became available that circumvents most of the above mentioned limitations (Coetzee and Glencross, 2017; Degandt et al., 2018). This fully automated device is able to load whole blood samples, process the sample (leukocyte staining, incubation and red blood cell lysis) and analyze leukocyte counts and receptor expression (Degandt et al., 2018). In the clinical setting the AQUIOS CL® flow cytometer is already used for analysis of CD4 T cell counts in human immunodeficiency virus (HIV)-positive patients (Coetzee and Glencross, 2017). Until now, fully automated flow cytometry has never been used for granulocyte or monocyte analysis despite the seemingly obvious advantages for phenotyping these complex and delicate cells. Fast and automated analysis minimalizes *ex vivo* manipulation and allows for timely processing. The AQUIOS CL® provides the means for steady and rapid analysis of *in vitro* activation. The aim of this study was to assess the AQUIOS CL® flow cytometer for analyzing activation of granulocytes and monocytes in a clinically applicable setting.

## 2. Materials and methods

### 2.1. Study participants

Blood samples were obtained from anonymous, healthy volunteers between the age of 18 and 65 years, male and female, who gave informed consent under protocols approved by the Medical Ethical Committee of the University Medical Center Utrecht. This study was conducted in accordance with the declaration of Helsinki.

### 2.2. Flow cytometry analysis by the fully automated AQUIOS CL® “Load & Go” flow cytometer

The AQUIOS CL® (Beckman Coulter Life Sciences, Miami, FL, USA) has one 488 nm diode laser, 2 light scatter channels (FSS and SSC), 5 fluorescence channels (FITC, PE, ECD, PE/Cy5(PC5), PE/Cy7(PC7)) and an electronic volume (EV) measure. The machine combines robotic automated sample preparation with automated analysis of cells using flow cytometry up to 22 samples per hour. A cassette filled with blood tubes is easily placed in the machine without the need for trained personnel. Hereafter, the device reads the barcodes on the blood tubes and saves the codes in the system. After automatic blood mixing, the blood tubes are cap-pierced and 43 µL is pipetted into a 96-deep wells plate. Thereafter the machine returns the blood tubes and proceeds with antibody staining. Consecutively, 18 µL of a monoclonal antibody mix bound to different fluorescent labels is transferred from a vial to the 96-wells plate. Multiple ready-to-use mixes of antibodies can be used for staining. After 15 min of incubation, the blood is lysed using 435 µL of lysing reagents A and B. Lysing reagent A is a cyanide-free lytic reagent that lyses red blood cells. Lysing reagent B slows the reaction caused by reagent A and preserves the white blood cells for measurement in the flow cell. Finally, 100 µL of prepared sample is aspirated for analysis. Absolute leukocyte count is based on an electronic-volume measurement. Gating is done automatically by the system's software and should only be revised when run notifications and flags are shown on the result screen. After a total of 100 tests the flow cytometer needs to be reloaded with new reagents. For daily quality control two commercial controls were used: the AQUIOS IMMUNO-TROL® Cells (Beckman Coulter) and the AQUIOS IMMUNO-TROL® Low Cells (Beckman Coulter). When working with neutrophils, the above mentioned method is very different and less time consuming than the conventional neutrophil analysis. An overview of the exact differences between the conventional and AQUIOS CL® method of neutrophil measurement is shown in supplement 1.

Originally, the AQUIOS CL® was designed for rapid analysis of CD4 cells. Only recently Beckman Coulter released the new AQUIOS CL® Designer Software 2.0, that allows tweaking of the device and thereby the introduction of this technique for analysis of granulocytes and monocytes. The designer software allows the user to change each step in the sample preparation process (volumes, incubation times, and mix cycles). In the analysis mode it is possible to set up user-defined protocols, acquisition templates, panels, compensation settings, and statistic / QC parameters. For this research purpose, customized antibody mixes were used, consisting of 1:10 CD35-FITC (clone J3.D3 Beckman Coulter Life Sciences, Miami, FL, USA), 1:14 CD16-PE (clone 3G8 Beckman Coulter Life Sciences, Miami, FL, USA), 1:10 CD62L-ECD (clone DREG56 Beckman Coulter Life Sciences, Miami, FL, USA), 1:10 CD11b-PC5(clone Bear1 Beckman Coulter Life Sciences, Miami, FL, USA) and 1:14 CD10-PC7(clone ALB1 Beckman Coulter Life Sciences, Miami, FL, USA).

The designer software allows up to four different protocols out of one blood tube. The machine can process a maximum 4 × 43 µL blood (4 wells) at a time and, depending on the protocol, different antibody mixes can be added. This was used to simultaneously analyze marker expression in the presence and absence of the bacterial/mitochondrial derived stimulus formyl-methionyl-leucyl-phenylalanine (fMLF, end concentration 10<sup>-6</sup> M).

### 2.3. Gating strategy

Cells were gated and analyzed using FlowJo® analysis software (Tree Star Inc., Ashland, Oregon). Monocytes and lymphocytes were gated from the original .lmd files on FSC/SSC. Neutrophils and eosinophils were identified based on FSC/SSC and CD16 expression (neutrophils CD16+ and eosinophils CD16-). For analysis the median

fluorescent intensity (MFI) in the five fluorescence channels of these gated cells was exported.

#### 2.4. Reproducibility experiments

Reproducibility tests were performed to test the stability of granulocyte and monocyte activation markers as measured by the AQUIOS CL<sup>®</sup>. To do so, blood of five healthy controls was tested in duplicate experiments. Blood was drawn in a sodium heparin tube (BD vacutainer, Franklin Lakes, New Jersey, USA) next to the AQUIOS CL<sup>®</sup> and immediately placed into the machine, situated in the emergency department, to minimize the time between blood drawing and sample preparation to < 2 min. This was repeated directly after the first analysis with the same blood sample. The interval between the two measurements was 2 min due to the processing time.

#### 2.5. Time experiments

To analyze the effect of bench time on granulocyte and monocyte activation, the expression of activation markers CD35 and CD11b was measured at different time points after blood drawing. For neutrophils the additional marker CD10 was analyzed. The first measurement was directly after the blood was drawn ( $T = 0$ ), repeated samples were taken at 1 h ( $T = 1$ ), 2 h ( $T = 2$ ), and 3 h ( $T = 3$ ) from the same blood tube and processed according to the same protocol. The blood tube was stored at room temperature next to the flow cytometer and the blood tube was inverted 2 times every 30 min to prevent sedimentation.

To test whether the activation of cells in time was specific for the tested individuals, after three weeks the same experiment was repeated with healthy control 1 to 5 at the same time during the day.

#### 2.6. Statistical analysis

Statistical tests were performed in GraphPad Prism 7<sup>®</sup> (Graphpad software, San Diego, CA). Data were presented as mean with 95% confidence interval (95%CI). The differences were assessed by repeated measures ANOVA test followed by the Dunnett's post-hoc test. To analyze the responsiveness of cells, the ratio between the MFI of the fMLF+ and fMLF- test was calculated. When the number of experiments was insufficient to test for normality, nonparametric tests were used. Results were regarded statistically significant when  $p < .05$ .

### 3. Results

#### 3.1. AQUIOS CL<sup>®</sup> analysis of activation marker expression on monocytes and granulocytes is highly reproducible

The first samples were analyzed with a 2 min time interval. These duplicate experiments are shown in Fig. 1 and demonstrate a high reproducibility of the measurements. The first measurement (duplo 1) was always slightly lower than the same sample measured after 2 min (duplo 2).

#### 3.2. A significant increase of activation markers on immune cells during time on the bench

##### 3.2.1. Neutrophils

Results regarding the expression of activation markers on neutrophils are shown in Fig. 2. Healthy controls (HCs) showed a very homogenous expression of activation markers at  $T = 0$  (95%CI (< 30%) in MFI; column 1, Fig. 2). Remarkably, activation markers were already significantly increased after 1 h ( $T = 1$ ) (mean (95%CI) CD35:  $2.2 \times (1.5 \times -2.5 \times)$   $p = .028$ , CD11b:  $2.5 \times (1.7 \times -3.1 \times)$   $p = 0.023$ , CD10:  $2.5 \times (2.1 \times -2.7 \times)$   $p = .009$ ) and a further increase in activation markers was observed after 2 and 3 h. The variation between donors increased significantly with each time points, resulting in a CI of

50% after 3 h. Some HCs showed a sixfold increase in activation markers, whereas other HCs only have activation markers that increased  $1.5 \times$ . Although there were distinct time-dependent levels of activation detected between HCs, the increase within HCs displayed a similar trend for all activation markers. Meaning that, if a HC showed a high upregulation of CD11b ( $6.0 \times$  up), CD35 ( $4.2 \times$  up) and CD10 ( $4.6 \times$  up) were also highly upregulated.

The expression of activation markers in the presence of fMLF (fMLF+) and the ratio fMLF+/fMLF- are depicted in columns 2 and 3 of

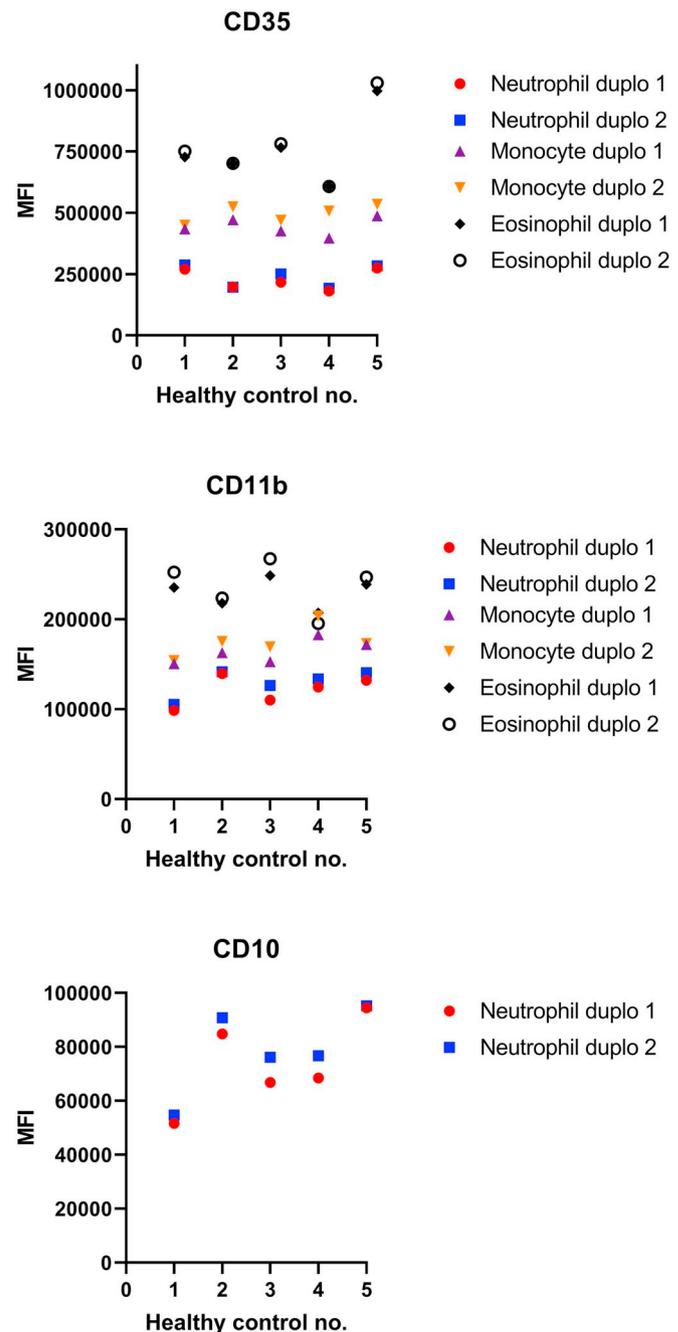


Fig. 1. Duplicate experiments in healthy controls. The median fluorescent intensities is depicted of the markers CD35, CD11b and CD10. Per antibody marker the duplicate experiments are shown per cell type. Duplo 1 is the measurement directly after blood drawing and duplo 2 is the same blood tube placed in the machine 2 min after the first one. All duplo 1 experiments have a slightly lower marker expression than the duplo 2 experiments. This indicates an increase in marker expression already in 2 min. The increase is most evident in neutrophils and monocytes.

# Neutrophils

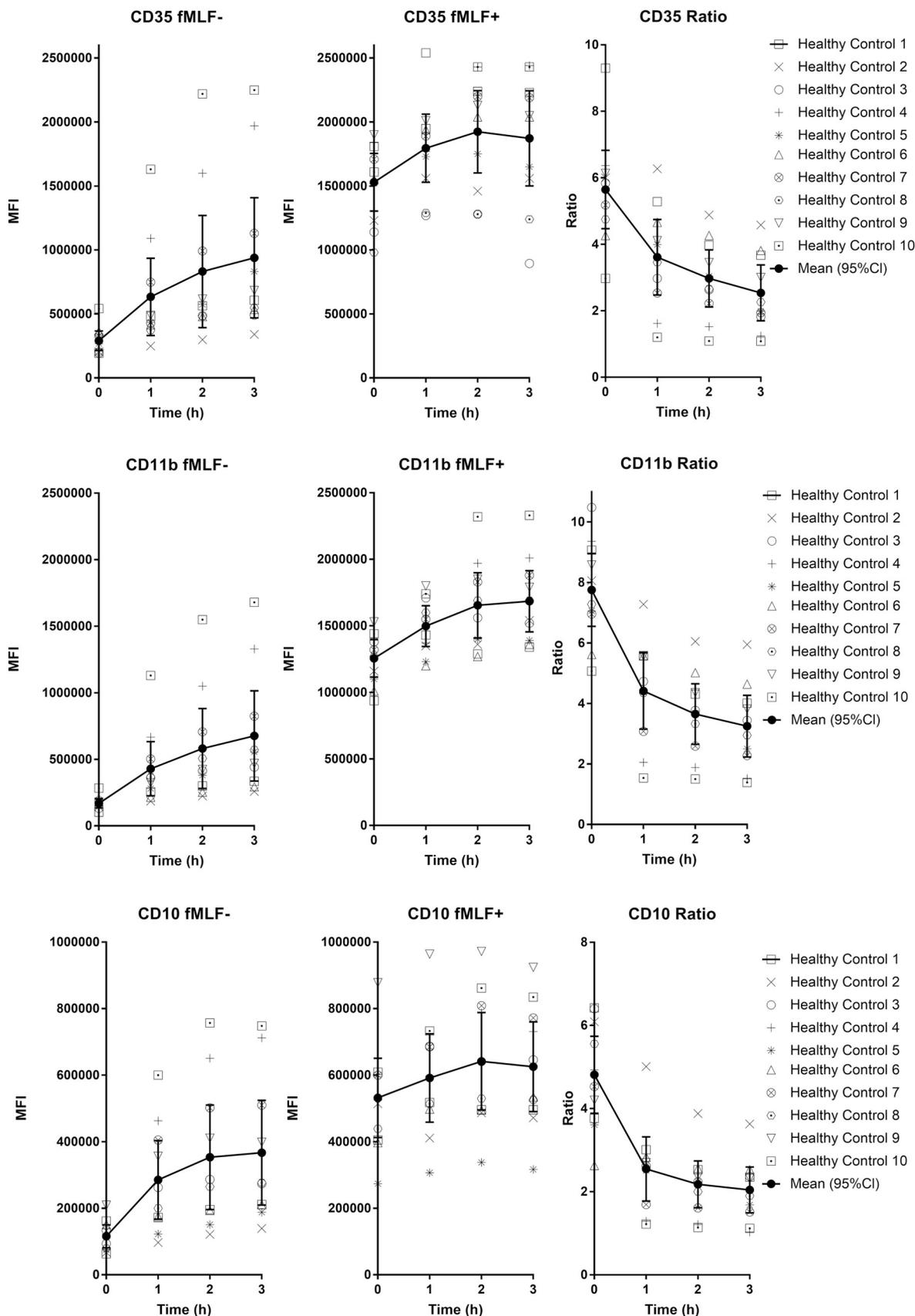


Fig. 2. Activation marker expression of neutrophils in the same blood tube over time. Median fluorescent intensities are depicted of the markers CD35, CD11b and CD10 on neutrophils. The first column shows the data in the absence of fMLF (fMLF-), the second column shows the data in the presence of 1  $\mu$ M fMLF (fMLF+) and the last column the ratio fMLF+/fMLF-.

Fig. 2, respectively. Overall a small increase was detected between  $T = 0$  and  $T = 1$  in the expression of the activation markers in the presence of fMLF:  $1.2 \times$  increase in CD35,  $1.2 \times$  in CD11b and  $1.1 \times$  in CD10. This increase was stabilizing at  $T = 2$  and  $T = 3$ . However, since the expression of activation markers in the absence of fMLF was significantly increasing, a clear decrease in the ratio fMLF+ /fMLF- was detected. The CD35 ratio decreased from 5.6 (4.5–6.8) to 3.6 (2.5–4.8) at 1 h and to 2.5 (1.7–3.4) at 3 h. This was even more evident in CD11b, where the ratio decreased from 7.7 (6.5–9.0) to 4.4 (3.1–5.7) to 3.2 (2.2–4.3). The CD10 ratio was also decreasing from 4.8 (3.9–5.7) to 2.0 (1.5–2.6) at 3 h.

### 3.2.2. Monocytes

Data regarding monocytes is shown in Fig. 3. Activation markers were already increased after 1 h of blood drawing (mean (95%CI) CD35:  $1.8 \times (1.3 \times -2.2 \times)$   $p = .058$ , CD11b:  $2.13 \times (1.6 \times -2.4 \times)$

$p = .025$ ). A further significant increase in activation markers was observed after 2 and 3 h. After 3 h CD35 is  $2.7 \times$  higher than  $T = 0$  and CD11b is increased by  $3.3 \times$ , compare to  $T = 0$ . The fMLF+ response of monocytes showed a similar pattern as found in neutrophils. The first hour the expression of CD35 and CD11b in the presence of fMLF was upregulated, with  $1.4 \times$  and  $1.3 \times$ , respectively. Thereafter the fMLF+ measurements were stabilizing. At  $T = 0$  the ratio for CD35 was 3.1 (2.5–3.7), which was decreasing to 2.7 (1.9–3.6) at  $T = 1$  and 1.7 (1.4–2.0) at  $T = 3$ . The ratio of CD11b was decreasing from 4.1 (3.0–5.1) at  $T = 0$ , to 2.8 (1.9–3.7) at  $T = 1$  to 1.7 (1.4–2.0) at  $T = 3$ .

### 3.2.3. Eosinophils

The eosinophil data is shown in Fig. 4. Activation markers were already mildly but significantly increased after 1 h of blood drawing (mean (95%CI) CD35:  $1.1 \times (1.1 \times -1.2 \times)$   $p = 0.005$ , CD11b:  $1.2 \times (1.2 \times -1.2 \times)$   $p = .005$ ). A further increase in activation markers was

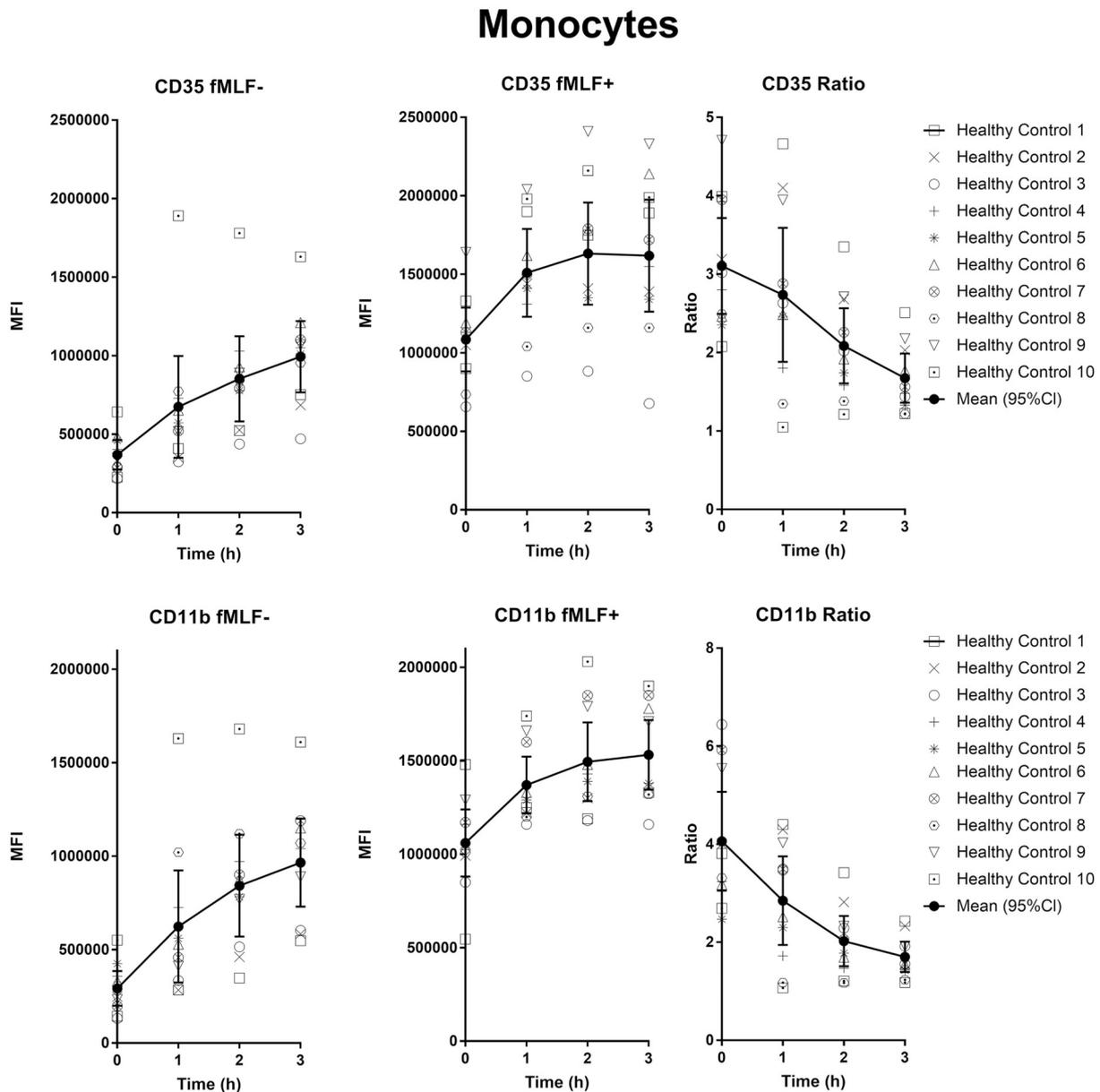
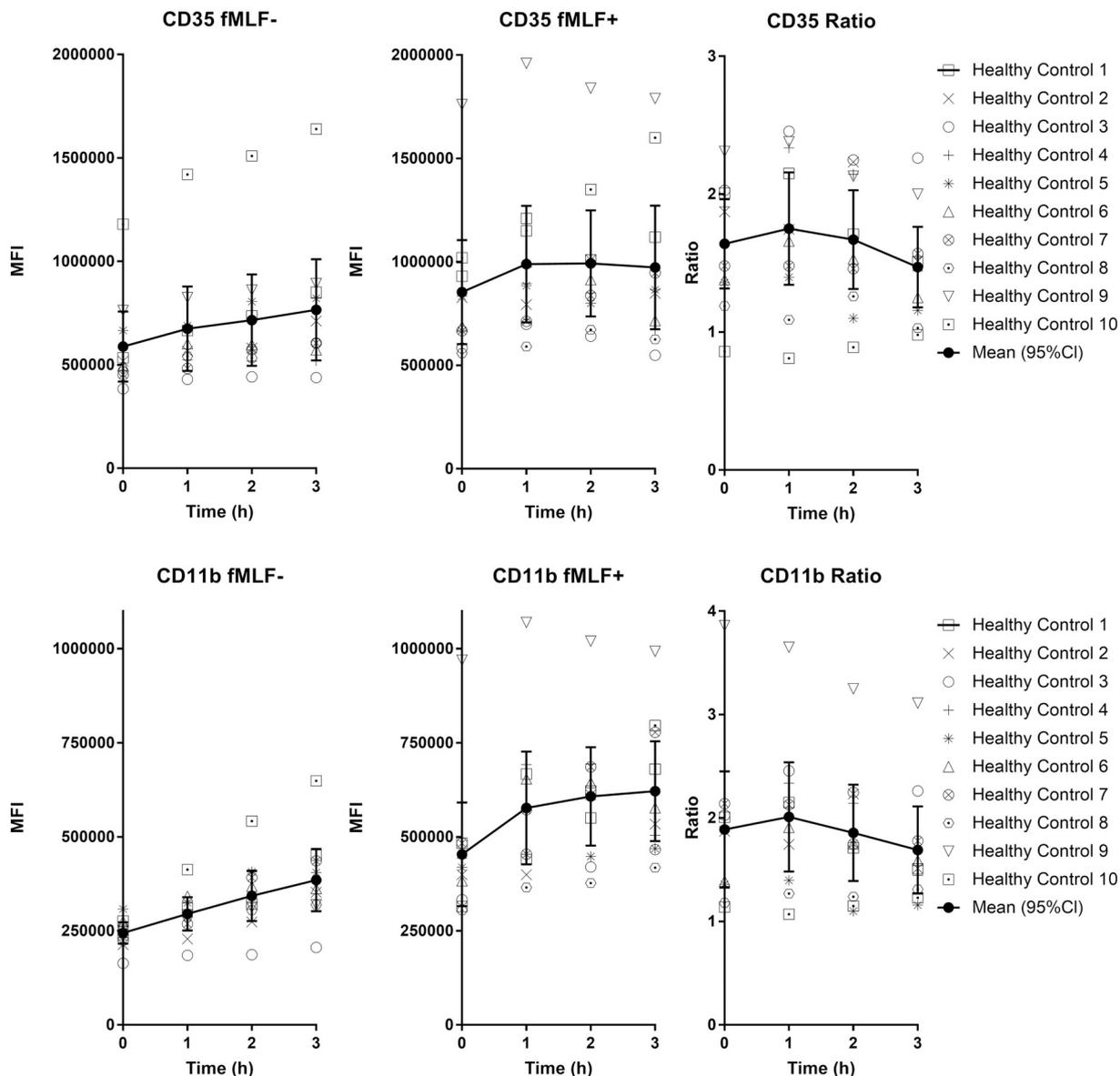


Fig. 3. Activation marker expression of monocytes in the same blood tube over time. Median fluorescent intensities are depicted of the markers CD35 and CD11b on monocytes. The first column shows the data in the absence of fMLF (fMLF-), the second column shows the data in the presence of 1  $\mu$ M fMLF (fMLF+) and the last column the ratio fMLF+ /fMLF-.

# Eosinophils



**Fig. 4.** Activation marker expression of eosinophils in the same blood tube over time. Median fluorescent intensities are depicted of the markers CD35 and CD11b on eosinophils. The first column shows the data in the absence of fMLF (fMLF-), the second column shows the data in the presence of 1  $\mu$ M fMLF (fMLF+) and the last column the ratio fMLF+/fMLF-.

observed after 2 and 3 h. The fMLF+ data on eosinophils showed a similar pattern as the neutrophils. The effect of time on CD35 and CD11b was minimal in fMLF+ experiments. In these experiments the ratio changed in time although to a much lesser extent compared with neutrophils.

### 3.3. The ex vivo time-dependent increase in activation markers is reproducible and seems donor specific

Blood was drawn after 3 weeks at the same day and time in the week. Results are shown in Fig. 5 for neutrophils, Fig. 6 for monocytes and Fig. 7 for eosinophils.

At baseline all healthy controls showed a similar level of activation markers at both sample moments. Healthy control 4 had the granulocytes and monocytes that were most easily activated during ex vivo

bench time both at the first venipuncture and the second venipuncture. When comparing the difference between neutrophil activation marker at T0 and T3 of the same donors at different venipuncture moments we see similar increases (CD35: 6.8 $\times$  vs. 5.7 $\times$ , CD11b: 8.8 $\times$  vs. 6.8 $\times$ , CD10: 5.8 $\times$  vs. 6.6 $\times$ ). Healthy control 2 and 3 are the least easily activated, with the smallest increase of activation markers at all time points. Of these individuals, HC3 showed the least increase in activation markers comparing T0 with T3 (CD35: 1.7 $\times$  vs. 2.5 $\times$ , CD11b: 1.8 $\times$  vs. 3.1 $\times$ , CD10: 2.1 $\times$  vs. 3.6 $\times$ ). All HCs approximately showed the same fMLF responsivity at both sample moments.

## 4. Discussion

This study showed that flow analysis with fully automated flow cytometry provides stable reproducible results for analysis of

**A.**

**Neutrophils baseline**

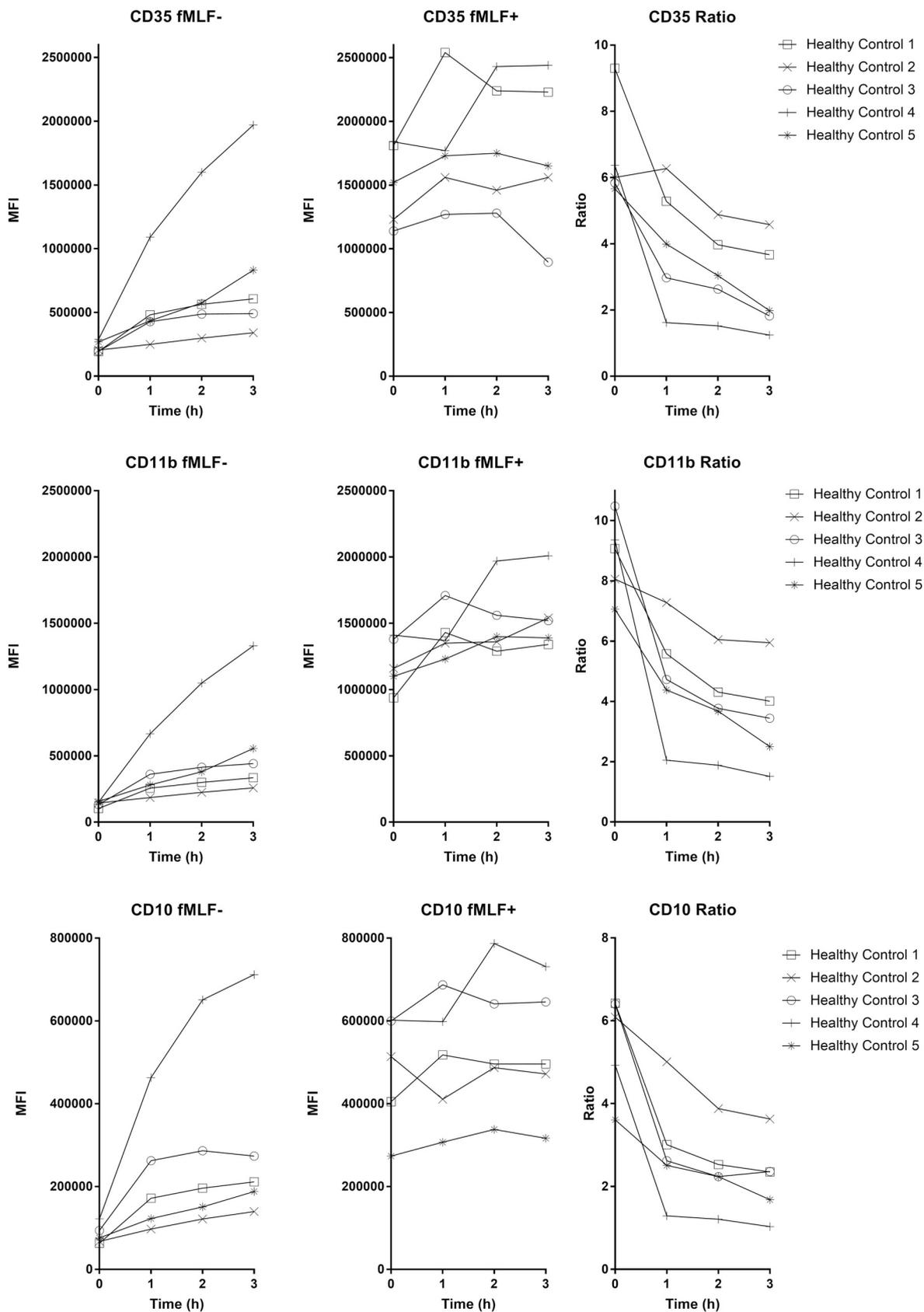


Fig. 5. Activation marker expression of neutrophils in the same blood tube over time for healthy controls 1 to 5. A shows the data sampled at baseline and B shows the data after three weeks. The median fluorescent intensities is depicted of the markers CD35,CD11b and CD10 on neutrophils. The first column shows the data in the absence of fMLF (fMLF-), the second column shows the data in the presence of 1  $\mu$ M fMLF (fMLF+) and the last column the ratio fMLF+ /fMLF-.

## B. Neutrophils after 3 weeks

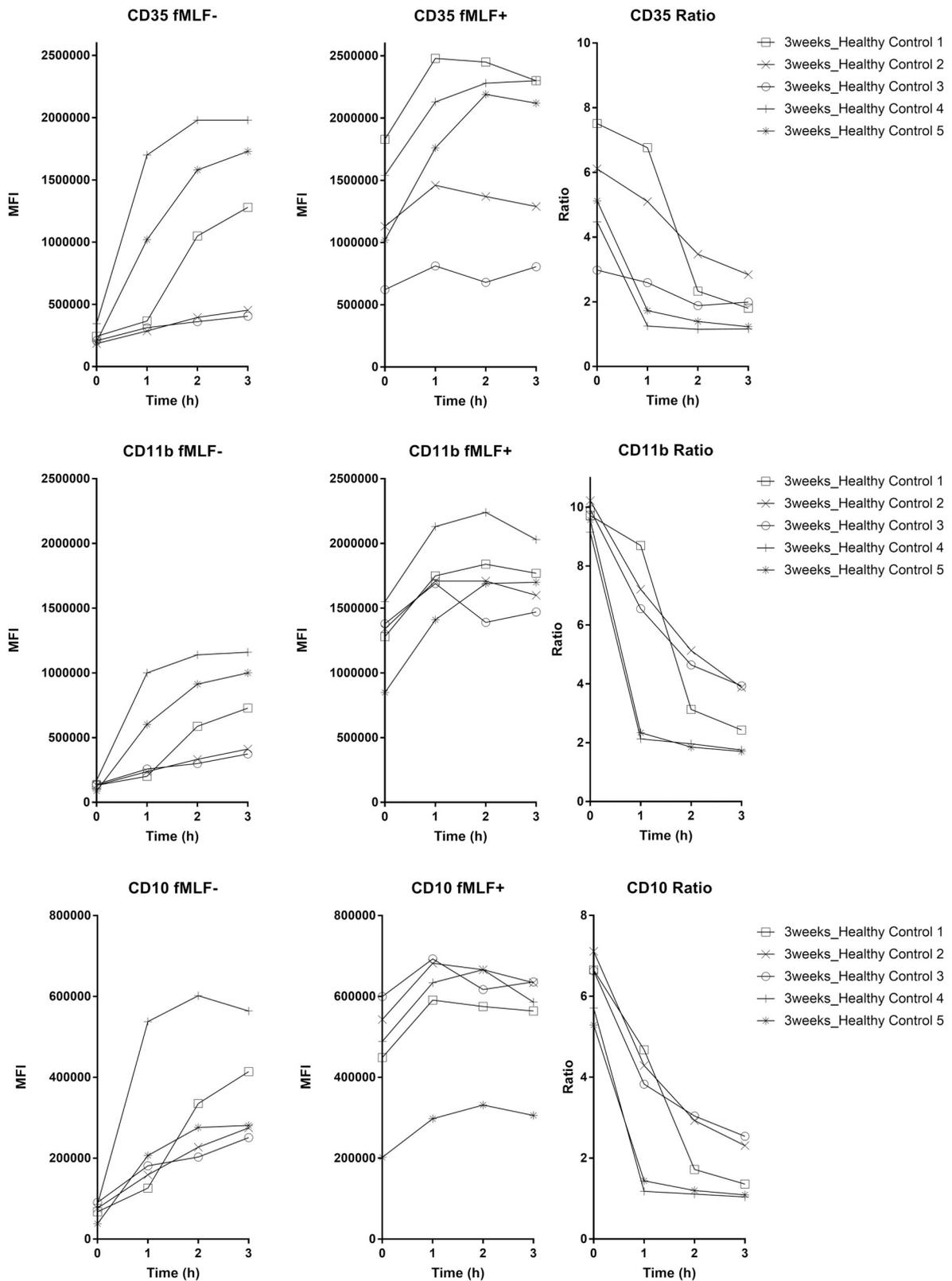
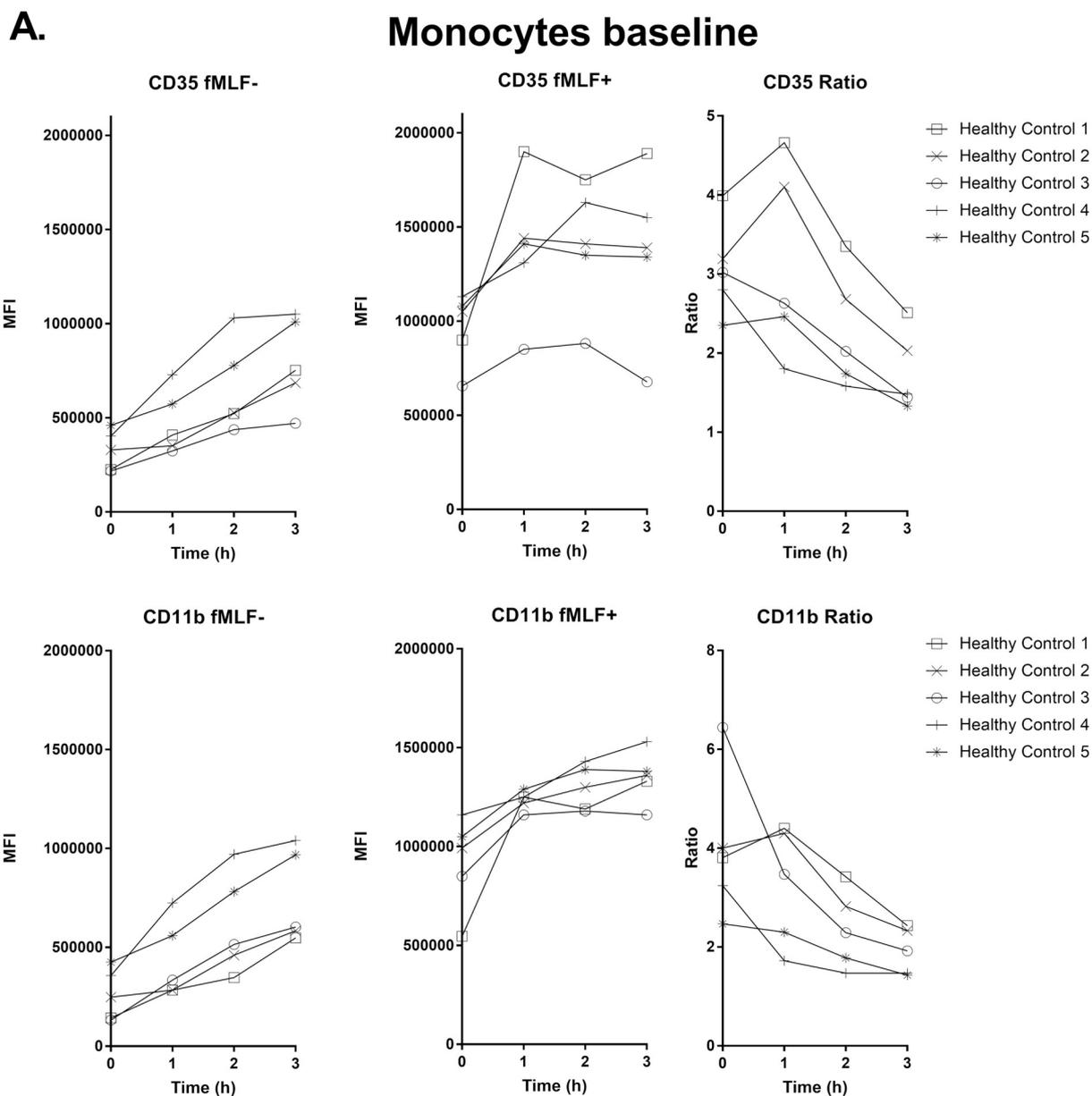


Fig. 5. (continued)



**Fig. 6.** Activation marker expression of monocytes in the same blood tube over time for healthy controls 1 to 5. A shows the data sampled at baseline and B shows the data after three weeks. The median fluorescent intensities is depicted of the markers CD35 and CD11b on monocytes. The first column shows the data in the absence of fMLF (fMLF-), the second column shows the data in the presence of 1  $\mu$ M fMLF (fMLF+) and the last column the ratio fMLF+/fMLF-.

expression of activation markers on phagocytes. Furthermore, we identified that expression of activation markers on granulocytes and monocytes was significantly upregulated during the bench time of the blood tube after blood drawing. Already 1 h after blood drawing a marked and significant increase of CD35 and CD11b on granulocytes and monocytes was found. This receptor expression increased further after 2 h and 3 h of bench time. In addition, we showed that this increase in expression of activation markers differed between healthy controls, meaning that the artifact caused by time delays in measuring activation receptor expression leads to different conclusions for different subjects. Interestingly, such variation between different donors was not seen for the marker expression of CD11b, CD35 and CD10 at T0. However, analysis of the same sample with the conventional processing steps, which takes 1 to 3 h, would have led to different conclusion.

To make firm conclusions regarding the determination of the activation status of the granulocytes and monocytes, it is important to show

the stability and reliability of the AQUIOS CL<sup>®</sup>. Degandt et al. evaluated precision, sample stability, inter-sample carry-over, linearity and interpanel consistency of the AQUIOS CL<sup>®</sup>, compared to a dual-platform method (Sysmex XE-5000 and BD FACSCanto-II) (Degandt et al., 2018). Variability between different tests was < 9.1% with dedicated control material. Interpanel inconsistency was 3.3% for relative values and 9.4% for absolute values. Method comparison showed good analytical correlation between AQUIOS CL<sup>®</sup> and a dual-platform method. So in conclusion, the study showed reliable results for adequate quantitation of T, B and natural killer lymphocytes. No results were mentioned for granulocytes or monocytes.

In our study we tested the stability of the data obtained with granulocytes and monocytes. We showed that neutrophil marker expression was very consistent in duplicate experiments. Therefore, it can be concluded that the increase of activation marker expression in granulocytes and monocytes is a true increase and not just the result of a putative variance of the machine.

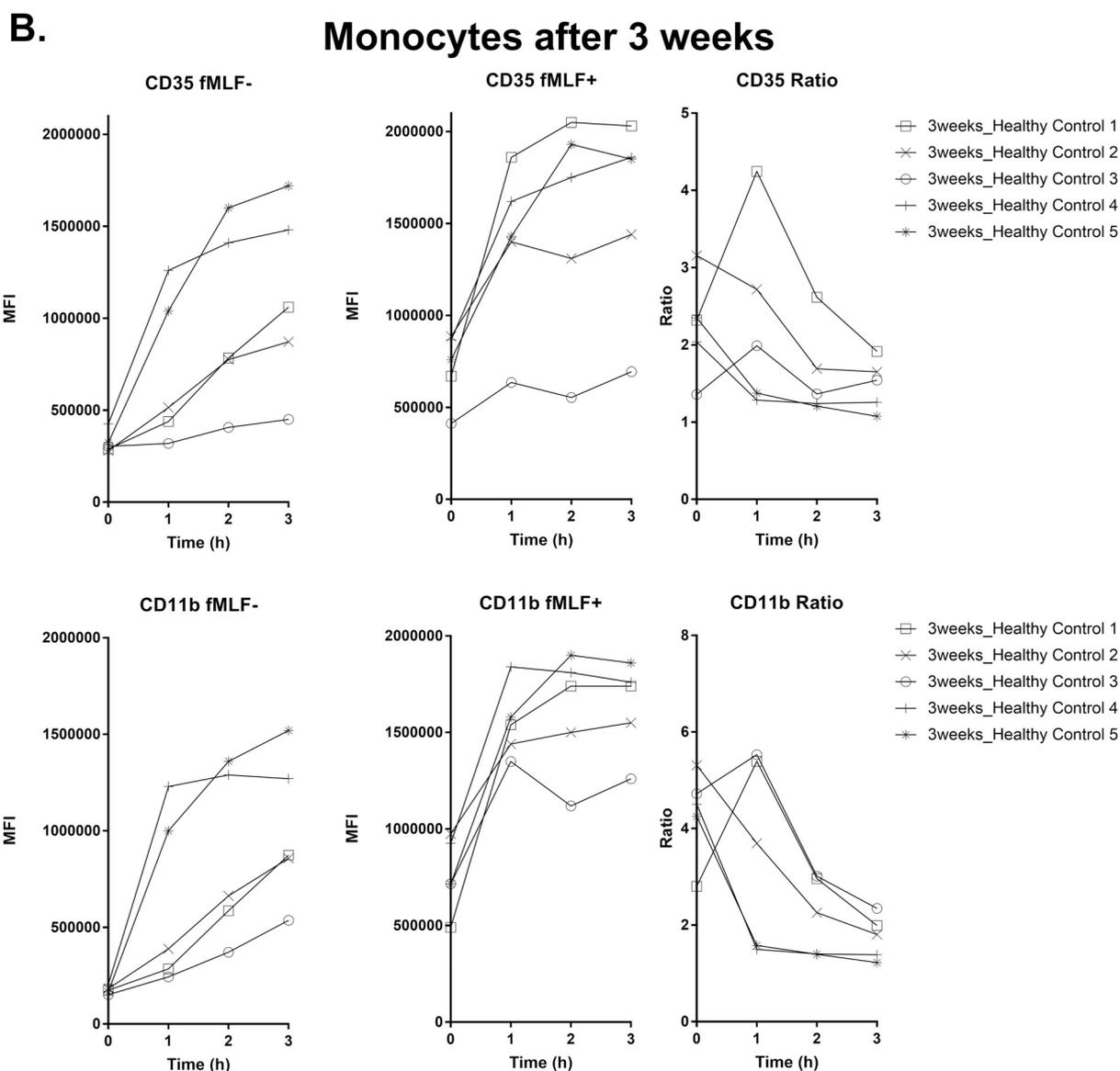


Fig. 6. (continued)

Several markers were used to study neutrophil and monocyte activation. Receptors that are known markers for neutrophil activation are the complement receptors type 1 (CR1/CD35) and 3 (CR3/Mac1/CD11b) (Sengelov, 1995). Both CR1 and CR3 are mainly expressed on phagocytes, such as macrophages, monocytes and granulocytes, but also in small numbers on lymphocytes. A reservoir of CR1 and CR3 receptors in phagocytes is present in cytoplasmic secretory vesicles which are translocated to the plasma membrane upon cell activation and are, therefore, used as activation markers to assess disease susceptibility and diagnose pathologies (Khara and Das, 2009; Sengelov et al., 1994; Springer et al., 1986; Vorup-Jensen and Jensen, 2018). However, for these clinical applications, accuracy is of utmost importance and our study shows that the effect of time should be taken into account to avoid drawing the wrong conclusions.

The receptor CD10, neprilysin, is widely used for diagnostic purposes in cancer and in the analysis of lymphocytes (Galy et al., 1995; McCluggage et al., 2001). Recently, studies started to focus on CD10 expression as maturation marker on neutrophils (Marini et al., 2017). There is also limited evidence available that demonstrates a higher neutrophil CD10 expression during systemic inflammatory diseases, suggesting that CD10 is also an activation marker (Eksioglu-Demiralp et al., 2001; Martens et al., 1999). In this study we showed that CD10

was upregulated by fMLF in a similar way as CD11b and CD35, which confirms that CD10 is also an activation marker.

Despite the fact that we only used four markers for this experiment, it is clear that a delay in flow cytometry analysis will cause problems for all markers associated with granulocyte and monocyte activation. Other markers associated with granulocyte and monocyte activation are: CD11c, CD32, CD66, CD63, CD64, CD55, CD14 and CD18 (Collison et al., 2015; Futosi et al., 2013). Our study indicates that a time delay in analysis of blood samples leads to artificial activation of phagocytes. It is even to be expected that such an artifact is more prominent in patients with inflammatory diseases as these cells are very sensitive for priming *in vivo* by inflammatory mediators (Miralda et al., 2017). However, it is not only inflammatory mediators that activate granulocytes and monocytes. In our experiments we used blood samples of healthy controls that were likely characterized by a minimal presence of inflammatory mediators. We still see a significant upregulation of activation markers over time, meaning that the cells became activated. One can only speculate on the underlying mechanism(s), but *ex vivo* manipulation, platelet-activation and release of their mediators and temperature changes have all been implicated in leukocyte activation (Kuijpers et al., 1991; Mani et al., 2004).

It is evident that measuring granulocytes and monocytes as soon as

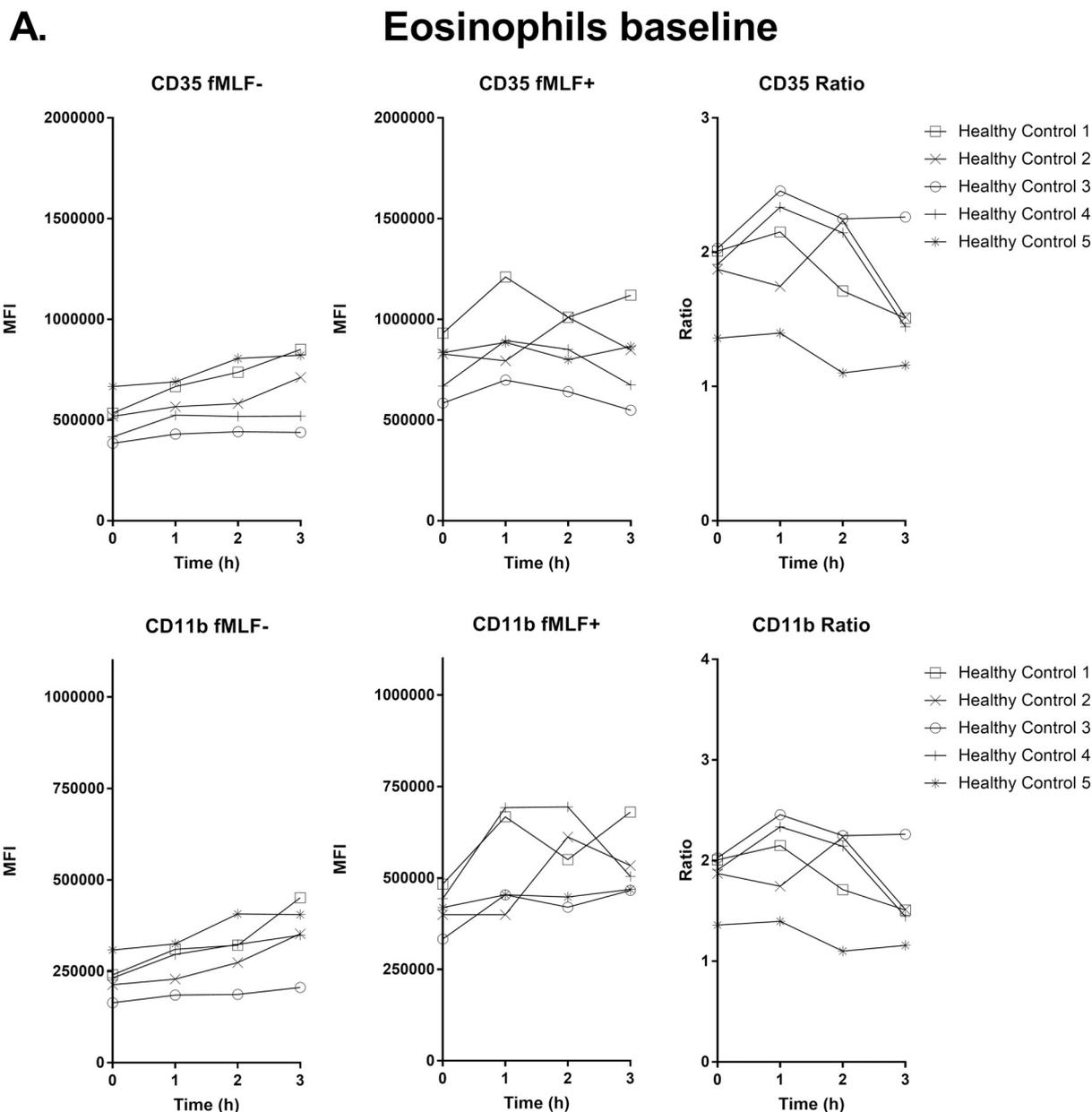


Fig. 7. Activation marker expression of eosinophils in the same blood tube over time for healthy controls 1 to 5. A shows the data sampled at baseline and B shows the data after three weeks. The median fluorescent intensities is depicted of the markers CD35 and CD11b on eosinophils. The first column shows the data in the absence of fMLF (fMLF-), the second column shows the data in the presence of 1  $\mu$ M fMLF (fMLF+) and the last column the ratio fMLF+ /fMLF-.

possible after blood drawing will mimic the *in vivo* marker expression the most. It is likely that granulocytes and monocytes already become slightly activated during the procedure of venipuncture. Although the AQUIOS CL<sup>®</sup> enables fast measurement, the measurement is still different from the *in vivo* situation. However, this automated immediate measurement is to our opinion the best available assay to determine the *in vivo* granulocyte or monocyte activation status. The fast flow analysis after blood drawing comes with several difficulties. The most important issue is the point-of-care placement of the flow cytometer as the analysis needs to be performed immediately. Therefore, without a directly available machine, such as in the present point-of-care situation, it is inevitable to end up with a delay in bench time and differences in artificial activation. This may result in wrong conclusions. An alternative might be specially developed ‘cell fix’ tubes or special reagents that are able to fix cells immediately, such as TransFix<sup>®</sup> from Cytomark that has proven its stability in lymphocytes (Canonico et al., 2004). However, several problems preclude the application of these methods for the

study of granulocytes. These problems include fixation-induced changes of epitopes modulating the binding of antibodies, difficulties in testing responsiveness of neutrophils for inflammatory mediators (e.g. fMLF) and interference with proper lysis of erythrocytes.

Point-of-care flow cytometry analysis is not very practical in many cases as often flow cytometry is carried out in a centralized flow facility that analyses all samples from the institution. All institutions that try to bring flow cytometry to monitor systemic inflammation face the difficulties discussed above in optimizing the workflow. In these cases, automated standardized “load & go” flow cytometry has great advantages. Standardization of the workflow without manual steps, gives faster and more accurate results. Automated preparation processes decrease the time of work for analysts and might spare laboratory expenses. Finally, the AQUIOS CL<sup>®</sup> has integrated software that is linkable to most of the electronic patient registry software packages, allowing the laboratory to provide analysis results quickly to clinicians.

In conclusion, this study showed that bench time of one hour

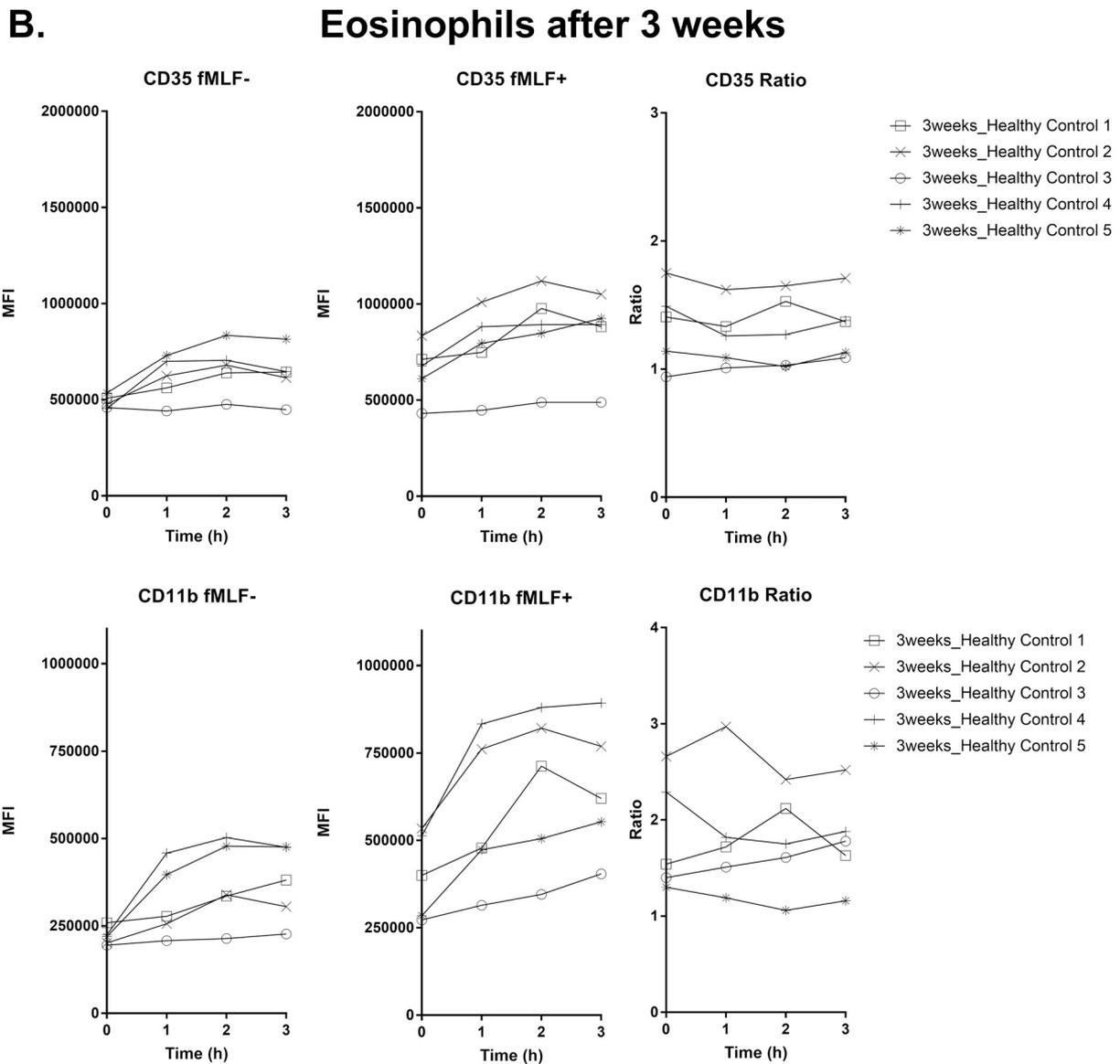


Fig. 7. (continued)

already leads to a significant upregulation and bigger variance in activation markers of granulocytes and monocytes. In addition, it is likely that automated flow cytometry reduces intra-assay variability in the analysis of activation markers on inflammatory cells. Therefore, we found that it is of utmost importance to perform immune activation analysis as fast as possible to prevent artificial activation, and drawing the wrong conclusions. Automated flow cytometry is able to reduce this analysis from 2 h to only 15 min without the need of dedicated personnel. This now allows fast and automated and even point-of-care flow analysis in blood samples in a variety of patient groups with the test result present 15–20 min after blood drawing.

**Acknowledgements**

This article was supported by a grant of NWO in the framework of the Startimpulse of the Dutch National Research Agenda (NWA).

**Declaration of Competing Interest**

The AQUIOS CL® “load & go” flow cytometer is provided by the company Beckman Coulter Life Sciences, Miami, FL, USA. All authors declare that there are no other competing interests.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jim.2019.112646>.

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