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Title: Detection of CD33 Expression on Monocyte Surface is Influenced by Phagocytosis and Temperature

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Abstract

CD33 is a myeloid-associated marker and belongs to the sialic acid-binding immunoglobulin (Ig)-like lectin (Siglec) family. Such types of receptors are highly expressed in acute myeloid leukemia, which could be used in its treatment. CD33 shows high variability in its expression levels with still unknown reasons. Here, we investigated the CD33 expression of monocytes in human blood samples processed at different temperatures and in dependence on their phagocytic activity against opsonized Escherichia coli. The samples were stained by fluorescently labelled anti-human CD14 to specify the monocyte population, anti-human CD33 antibodies to evaluate CD33 expression and analyzed by flow cytometry and confocal laser scanning microscopy. In blood samples kept at 37°C or first pre-chilled at 0°C with subsequent warming up to 37°C, the percentage of CD33-positive monocytes as well as their relative fluorescence intensity was up-regulated compared to samples kept constantly at 0°C. After exposure to E. coli the CD33 relative fluorescence intensity of the monocytes activated at 37°C was 3 to 4 times higher than that of those cells kept inactive at 0°C. Microscopic analysis showed internalisation of CD33 due to its enhanced expression on the surface

followed by engulfment of E. coli.

Keywords: CD33; expression; pre-analytical conditions; internalisation

Changelog
All figures were modified in agreement with the requests.

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Detection of CD33 expression on monocyte surface is influenced by phagocytosis and temperature

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Abstract. CD33 is a myeloid-associated marker and belongs to the sialic acid-binding immunoglobulin (Ig)-like lectin (Siglec) family. Such types of receptors are highly expressed in acute myeloid leukemia, which could be used in its treatment. CD33 shows high variability in its expression levels with still unknown reasons. Here, we investigated the CD33 expression of monocytes in human blood samples processed at different temperatures and in dependence on their phagocytic activity against opsonized *Escherichia coli*. The samples were stained by fluorescently labelled anti-human CD14 to specify the monocyte population, anti-human CD33 antibodies to evaluate CD33 expression and analyzed by flow cytometry and confocal laser scanning microscopy. In blood samples kept at 37°C or first pre-chilled at 0°C with subsequent warming up to 37°C, the percentage of CD33-positive monocytes as well as their relative fluorescence intensity was up-regulated compared to samples kept constantly at 0°C. After exposure to *E. coli* the CD33 relative fluorescence intensity of the monocytes activated at 37°C was 3 to 4 times higher than that of those

cells kept inactive at 0°C. Microscopic analysis showed internalisation of CD33 due to its enhanced expression on the surface followed by engulfment of *E. coli*.
Key words: CD33 — Expression — Pre-analytical conditions — Internalisation

Introduction

Clusters of differentiation (CDs) are receptors or surface markers used to classify cell type and maturation stage of leukocytes as well as other associated cells by staining with specific antibodies. CD antigens as receptors and ligands accomplish a variety of critical functions in the immune response such, cell signal cascades and cell adhesion (Zola et al., 2007). In certain circumstances CD antigens are expressed in some specific developmental stage or under some environmental and experimental conditions with different expression level. Consequently, the patterns of expression of cell surface CD antigens are promising goals for diagnostic and therapeutic clinical applications and research of different types of diseases such as cardiovascular disease, cancer, immunotherapy, and drug targeting (Golay et al., 2000; Sakamoto, et al., 2009; Woolfson et al., 2006).

CD33, a 67 kDa type I transmembrane cell surface glycoprotein receptor, belongs to the sialic acid-binding immunoglobulin-like lectins (Siglecs, Siglec-3) family. Structurally, CD33 contains a V-set domain, a C2-set domain, and a transmembrane region followed by immunoreceptor tyrosine-based inhibitory motif (ITIM) and ITIM-like motif (Laszlo et al., 2014). CD33 is expressed at high level on monocytes and macrophages, but at low level on mature granulocytes (Andrew et al., 1983; Freeman et al., 1995). Besides, CD33 is an accepted surface marker to identify monocytes (Terstappen et al., 1990). However, despite the crucial role of CD33, little is known of its function in myeloid cells, except that it may acts as an inhibitory molecule on the innate immune cells to mediate the cell–cell interaction and to inhibit normal functions through a reducing effect on tyrosine kinase-driven signaling (Crocker et al., 2008; Paul et al., 2000). Recent studies have shown that antibodies specific to CD33 possessed an ability to activate cytokine secretion by monocytes, suggesting a potential role of CD33 molecule in the cytokine responses of the immune system (Lajaunias et al., 2005). In addition, it has been noted that CD33 acts as an inhibitory factor on dendritic cell differentiation (Ferlazzo et al., 2000). Dendritic cells are antigen presenting cells which mainly interact with the adaptive immune system whereas monocyte derived macrophages are part of the innate immune response. CD33 up-regulation might inhibit dendritic cell differentiation when faced with a high number of pathogens (such as opsonized bacteria) to ensure the availability of these cells for phagocytosis and elimination of these pathogens. Previous studies have also shown this up-regulation of CD33 upon chronic obstructive pulmonary disease (COPD) patients was higher than that in the normal control group; however, no statistically significant differences were found between control group and patients (Zhang et al., 2013).

Moreover, the knowledge of CD33 expression levels could offer promising therapeutic strategies for certain diseases. Previous studies reported a correlation between the expression of CD33 and Alzheimer's disease and that the inhibition of CD33 may be a promising therapeutic target for this disease (Hooli et al., 2016; Jiang et al., 2014). Furthermore, the level of CD33 has been shown to be associated with the disease prognostic factors for acute myeloid leukemia (AML) and may thus serve as an attractive candidate for antibody-based therapeutic (Cowan et al., 2013; Krupka et al., 2014; Laszlo et al., 2014).

It is well established that several factors such as purification methods, storage and incubation temperature, or specimen age and anticoagulants affect the antigen expression levels of certain cell surface proteins of leukocytes. Some leukocyte surface markers (CD11a,b,c, CD18 and CD35) can be increased by handling procedures and temperature changes (Fearon et al., 1983; Forsyth et al., 1990; Lundahl et al., 1995; Miller et al., 1987), whereas these factors have no effect on other antigens for example CD15s, CD44, or CD62L (Youssef et al., 1995). Nevertheless, it was also reported that preparation procedure at higher temperature decreased the expression of CD62L (Lundahl et al., 1995; Stibenz et al., 1994).

Some studies found that besides temperature, phagocytosis may also influence the expression of antigens, suggesting that these changes may be caused by inflammation due to immunological response following phagocytic activity. For instance, the expression of CD11b and CD35 is increased (Repo et al., 1995), while CD64 and CD88 were not altered (Furebring et al., 2004) but CD14 is decreased in lipopolysaccharide (LPS)-stimulated monocytes (Jorgensen et al., 2001). Hence, the knowledge about up- or down-regulation of surface markers might be useful for therapeutic concepts.

To date, only a few research reports give detailed information on sample handling for the investigation of CD33 expression. The influence of temperature and phagocytosis on up- or down-regulation of CD33 expression should be clearly understood to support existing or future diagnostic and therapeutic approaches. Therefore, in the current study we investigated the effect of temperature as well as the presence of phagocytosis activating agents, *E. coli*, on the expression level of monocytes.

Materials and Methods

Materials

Phosphate buffered saline (PBS) pH 7.4 stock solution (10×) was purchased from Fisher Scientific (Pittsburgh, PA). NH_4Cl, $NaHCO_3$, EDTA were purchased from Sigma. PerCP/Cyanine5.5 anti-human CD33 Antibody, Mouse IgG1, κ, clone WM53 and clone P67.6 were purchased from BioLegend (San Diego, CA). Isotype control (non-specific isotype control antibody) PerCP/Cyanine5.5 Mouse IgG1, κ , Isotype Ctrl Antibody was purchased from BioLegend (San

Diego, CA). Alexa Fluor® 488 anti-human CD14 antibody (Clone M5E2) was purchased from BD Pharmingen (San Diego, CA). Lithium heparin vacutainers (34 I.U.) were purchased from Becton Dickinson (Plymouth, UK). Phagotest™ and Phagoburst™ kit were purchased from Glycotope-Biotechnology (Heidelberg, Germany). All chemicals used for experimental work were of analytical grade.

Blood collection, preparation and leukocyte staining

Freshly withdrawn venous blood anticoagulated by lithium heparin was collected from healthy volunteers. Informed consent was obtained from all donors in written form. The blood samples were withdrawn in accordance with the transfusion law of Germany. The use of donor blood samples for scientific purposes was approved by the ethics committee of the Charité – Universitätsmedizin Berlin (# EA1/137/14). Two tubes of blood were collected at the same time: one sample was immediately transferred to an ice bath (0 °C), and the other sample was taken into a water bath and kept at 37 °C. Figure 1 shows the experimental design. The samples were handled in three different ways. Whole blood was aliquoted into 50 µl samples in three separate tubes. One tube was maintained always on ice (0 °C). A second tube was chilled for 10 min at 0 °C and then transferred to a water bath for 10 min at 37 °C. The third tube was placed immediately and maintained in the water bath at 37 °C.

At the end of the incubation period, all samples were placed in the ice-bath, and washed with ice-cold PBS. The cells were re-suspended and then incubated with anti-CD14 and anti-CD33 antibody using concentrations suggested by the manufacturer with a concentration of 16 µg/ml for 30 min at 0 °C in darkness. Erythrocytes were lysed with ammonium chloride solution (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) for 15 min. The cells were washed twice and re-suspended in ice-cold PBS and then immediately analysed by flow cytometry

Phagocytosis of bacteria at different temperatures

Phagocytosis of non-labeled E. coli

Opsonized *E. coli* ($1–2 \times 10^9$ bacteria *per* ml, Phagoburst kit^{TM}) was used to examine the engulfment of bacteria. The samples were handled in the same way as in the temperature experiments. One tube was maintained always on ice the other tube was pre-chilled for 10 min and then transferred from the ice bath to the water bath (37 °C) and warmed up for 10 min at 37 °C after adding 10 µl of non-labeled opsonized *E. coli*. In parallel, the samples kept at 37 °C were incubated for 10 min with 10 µl of *E. coli*.

After 10 min, engulfment and uptake were stopped by cooling to 0 °C and washing with ice cold PBS. Subsequently, cells were stained with anti-CD14 and anti-CD33 antibody with a concentration of 16 µg/ml for 30 min at 0°C in darkness, followed by erythrocyte lysis using

ammonium chloride lysing solution for 15 min. Cells were washed and re-suspended in PBS and immediately analysed with the flow cytometer.

Phagotest of FITC-labeled opsonized E. coli

Phagotest™ kit was used to confirm healthy phagocytotic activity of monocytes. Manufacturer's instructions were partially modified: all reactions were performed with half of the volume, samples were incubated at two different temperature conditions, lysing solution was changed to ammonium chloride lysing solution, and DNA was not stained.

Internalisation of CD33 during phagocytosis

To examine the internalisation of CD33, 50 µl of blood were mixed with anti-CD14 and anti-CD33 antibody with a final staining concentration of 16 µg/mL for 30 min at 0 °C in darkness. Then 10 µL opsonised *E. coli* (1–2 × 109 bacteria *per* ml) was added and incubated at 37 °C or an ice bath (0 °C) for negative controls for 10 min.

After 10 min, engulfment and uptake were stopped by cooling to 0 °C and washing with ice cold PBS. Subsequently, ammonium chloride lysing solution was added and incubated on ice for 15 min. Cells were washed and re-suspended in PBS and immediately analysed with the flow cytometry.

Flow cytometry

The leukocytes were analysed by flow cytometry (FACS-Canto II, Becton and Dickinson, Franklin Lakes, NJ, U.S.A.) after diluting in PBS with ratio of 1:40 (Tölle et al., 2010; Zhao et al., 2017). 10,000 total events from each tube were collected. Monocytes, granulocytes, and lymphocytes were identified based on their forward and sideward scatter (FSC and SSC, resp.) characteristics. Then, the additional staining with anti-CD14 was gated out to identify monocyte population. Subsequently, positively stained CD33 cells were determined in the PerCP/Cy5.5 fluorescence channel as relative median fluorescence intensity (RFI). Data were analysed using the FlowJo v10 software (Tree Star, Ashland, OR).

Confocal laser scanning microscopy (CLSM)

Non-labeled (control), anti-CD14, and anti-CD33 labeled samples were investigated using a confocal laser scanning microscope (CLSM; ZeissLSM 510 meta, Zeiss MicroImaging GmbH, Jena, Germany) equipped with a 100 × oil immersion objective, with a numerical aperture of 1.3. Images of the samples were prepared in transmission and fluorescence mode with fluorescence excitation at 488 nm for both FITC as well as PerCP/Cy5.5, a band pass filter (513–556 nm) for FITC emission and a 650 nm long pass emission filter for PerCP/Cy5.5. Cells stained with anti-CD33 and anti-

CD14 antibodies were identified as monocytes. The fluorescence distribution inside the monocytes before and after stimulation with *E. coli* was investigated by analysis of z-stacks applying the LSM 510 software.

Statistical analysis

Analyses and graphs were performed using GraphPad Prism 6 software (GraphPad, San Diego, CA). Statistical analysis was performed using one-way analysis of variance followed by the Tukey multiple comparison test to determine the significance of particular comparisons. Two-way analysis of variance was used to determine significance in temperature and phagocytosis factors. Significance was defined as p-value of < 0.05, and is presented as * $p < 0.05$, ** $p < 0.01$, or **** $p < 0.0001$.

Results

The monocyte, granulocyte and lymphocyte populations in all samples could be clearly identified by flow cytometry in the SSC/FSC dot plots. The monocytes are then defined by sequential gating on all CD14-positive leukocytes in light scatter plots. More than ninety percent of the CD-33 positive were CD14-positive cells. The CD33 labeling was highly specific for the monocytes (Fig. 2).

Temperature-dependent influence in CD33 expression on monocyte surface

The influence of temperature treatment on CD33 expression of monocytes was investigated as shown in Figure 1. Monocytes maintained at 0 °C at all stages of preparation were defined as the reference levels of expression of CD33.

Monocytes that were pre-chilled and subsequently warmed up and those maintained at 37 °C throughout their preparation showed a significantly higher RFI of CD33 compared to the reference cells maintained at 0 °C. There was slightly lower, but not significantly different expression level, for cells cooled at 0 °C and subsequently warmed to 37 °C than those cells maintained at 37 °C at all stages of preparation (Fig. 3a, solid bars).

These results were independent on the used monoclonal antibody against CD33 clone WM53 and P67.6, respectively. Fig 3c shows the histograms of four different sample types at the investigated temperatures. Isotype staining as well as samples without staining provide the same very low fluorescence intensity under all conditions. The fluorescence intensities of the stained samples (clone WM53 and clone P67.6) show no significant differences.

Changes in CD33 expression due to phagocytosis of E. coli

The ability of monocytes to perform phagocytosis was tested for each donor in parallel applying the standard procedure of FITC-labeled *E. coli* as recommended in the Phagotest™ kit instructions. Percentages of phagocytizing monocytes and granulocytes and mean fluorescence intensity upon FITC-labeled *E. coli* treatment from healthy donors is given in Table 1. After incubation with FITC-labeled *E. coli* a strong increase in both percentages of phagocytosis and mean fluorescence intensity was observed, confirming the ability of monocytes and granulocytes to perform phagocytosis. The fluorescence signal of the phagocytosis activated by FITC-labeled *E. coli* was significantly increased in the FITC-A channel of the monocyte (Fig. 4a) and granulocyte (Fig. 4b) population.

Phagocytosis of *E. coli* induced a significant increase in monocyte expression of CD33 of cells maintained at 37 °C, or cooled to 0 ° C and subsequently warmed to 37 °C in comparison with the reference population of cells held at 0 °C which did not phagocytose *E. coli* (Fig. 3b).

The results clearly show that together with temperature, phagocytosis has an augmented effect on the expression of CD33. Monocytes held permanently at 37 °C and incubated with *E. coli* up-regulate the expression of CD33 during phagocytosis in contrast to cells chilled throughout at 0 °C. An additional large increment of CD33 expression occurs when the cells interact with *E. coli* (Fig. 3a, open bars).

Internalisation of CD33 during phagocytosis

The influence of phagocytosis was further investigated comparing the CD33 staining in samples where the antibody was applied after stopping the phagocytosis of *E. coli* (shock-cooling at 0 °C) with the staining of samples where the antibody was added before phagocytosis activation (warming up to 37 °C). In parallel to the quantitative determination of CD33 expressing monocytes by flow cytometry, we studied also the distribution of fluorescence in the cells by CLSM.

Double staining with FITC-labeled Anti-CD14 and PerCP-Cy5-5-A-labeled Anti-CD33 does not allow the use of FITC-labeled *E.coli*. Therefore the ability of monocytes to perform phagocytosis was tested for each donor in parallel applying the standard procedure of FITC-labeled *E. coli* (Fig. 5a). Interestingly, by performing double staining, we found that in monocytes the CD14 co-localised with CD33. It can be seen that the monocytes stained after stopping the phagocytosis exhibit a relatively weak fluorescence signal with a distribution mainly on the cell surface (Fig. 5b). In contrast, the samples stained before adding *E. coli*, the fluorescence signal was significantly higher and fluorescence was observed by microscopy not only on the surface but also inside the cells (Fig. 5c).

The flow cytometry measurements of the blood samples activated for phagocytosis with *E. coli* showed a slightly enhanced fluorescence signal in the PerCP-Cy5.5 channel of the granulocyte population. Fluorescence from CD33-positive granulocytes could only be detected when anti-CD33 was added before performing phagocytosis in contrast to the samples where the staining was

performed after stopping phagocytosis (Fig. 6b). Since this population was also clearly negative for staining with anti-CD33 without *E.coli* stimulation, a certain non-specific binding of the PerCP-Cy5.5-stained CD33 antibody on the opsonized *E. coli* was assumed. To confirm this, we incubated the opsonized unlabeled *E. coli* used for the activation of phagocytosis with the PerCP-Cy5.5-anti-CD33 and measured the fluorescence signal of the bacteria in the PerCP-Cy5.5 channel with the same settings as for the cells (Fig. 6a). The obtained fluorescence signal was similar to the signal measured in the PerCP-Cy5.5 for granulocytes stained before phagocytosis confirming that this signals is due to the engulfed bacteria with some antibody bound on them. In contrast, the fluorescence signal of the monocyte population is at least one order of magnitude higher in the same sample (Fig. 6c).

Discussion

The expression of CD surface antigens may change in response to several conditions in varying degrees. The expression of CD33 has long been recognised as a monocyte lineage marker (Terstappen et al., 1990), which helps to detect monocytes by flow cytometry.

Our results have shown that the RFI of CD33-positive monocytes which had been first cooled and then re-warmed was up-regulated compared with those held throughout at 0 °C, but not significantly different from those handled throughout at 37 °C. To our knowledge, the effect of temperature on the expression levels of CD33 had not been evaluated yet. It has been previously shown that warming of neutrophils from 0 to 37 °C (Berger et al., 1984), or of neutrophils or monocytes from 4 to 20 °C (Jämsä et al., 2011; Lundahl et al., 1995), or of monocytes from 4 to 37 °C (Fearon et al., 1983; Miller et al., 1987) as well as maintaining throughout at 37 °C (Jämsä et al., 2011) strongly up-regulates antigen surfaces, but holding throughout at 4°C the changes during storage are lower. Our results are in agreement with these findings and show that monocytes undergo similar changes. The molecular mechanism underlying up-regulation are still unclear. One possibility is that the rapid increase in surface presentation of CD33 on stimulated monocytes may be caused by a translocation of intracellular pool to the cell surface (Siddiqui et al., 2017). It has been reported that the stimulation of LPS results in increased surface expression of CD11b, and CD35 on monocytes, suggesting that these rapid changes may be caused by the inflammatory response (Furebring et al., 2004). In our study, an increase in CD33 expression upon *E. coli* stimulation was found. Therefore, up-regulation in CD33 may be involved in the inflammatory response, which has been shown to follow the initial systemic pro-inflammatory reaction. CD33 has a high expression on monocyte surface as well as in an internal compartment after stimulation of formylated peptides (fMLP), a bacterial-derived peptide. This could affect the expression of CD33 on the cell surface in response to an inflammatory stimulus (Siddiqui et al., 2017). It is also found that the high antigen induction on the cell surface upon *E. coli* activation may imply the preformed

intracellular pool of surface antigen which was rapidly translocated to the surface upon activation of these cells (Siddiqui et al., 2017). However, how LPS-elicited cell signaling regulates CD33 surface expression is not clear.

The results presented here are in contradiction with previous studies which have published that down-regulation of CD33 expression was observed when monocytes were activated by LPS (Lajaunias et al., 2005; Siddiqui et al., 2017). However, LPS is only one component of the gram-negative bacterial cell wall. Our work was performed with opsonized *E. coli* which presents a cellular pathogen. The immune system activation and subsequent responses to LPS and *E. coli* may therefore differ. Another observation, in our experiments, CD33 expression increases considerably within a very short time after contact with bacteria. This is in disagreement with the previously reported results, where the incubation time of LPS was up to 2 h (Lajaunias et al., 2005; Siddiqui et al., 2017). It appears that a longer time period is required to change the CD33 expression profiles by LPS. Based on the raising level of CD33 expression after exposure to *E. coli*, revealed significantly altered expression levels in monocytes might be a key element for diagnosis of septic shock. However, the outcomes should be further verified by higher number of blood samples from healthy donors and sepsis patients.

It has been shown that the engagement of both surface CD33 antigen and anti-CD33 antibody, induces receptor-mediated endocytosis (Walter et al., et al., 2008), resulting in CD33 internalisation of the antigen/antibody complex into the cells (Audran et al., 1995). This process may reduce the CD33 presented on the cell surface, but it is continuously re-expressed (Van Der Velden et al., 2001). The mechanism of action indicated that the presence of ITIM in the intracellular domain of CD33 is critical for the antibody-mediated CD33 internalisation (Walter et al., 2012; Walter et al., 2008). Intracellular trafficking of CD33 shows that it undergoes endocytosis *via* clathrin-mediated uptake and further traffics to endosomes and processes in lysosomes (Walter et al., 2012). Moreover, phosphorylation-dependent ubiquitylation of CD33 decreased the cell surface expression and increased the rate of CD33 internalisation (Walter et al., 2008). As a Siglec family member, CD33 has lectin-like recognition molecules which is one of the pattern-recognition receptors (PRRs) (Vasta, 2009). These receptors recognise pathogen-associated molecular patterns (PAMPs) from microbial pathogens in the first step of phagocytic process. An immune response is then triggered when PAMPs are recognised. There are some studies mentioned that the treatment of monocytes with anti-CD33 antibodies induced the production of pro-inflammatory cytokines (IL-1β, IL-8 and TNF-α) (Lajaunias et al., 2005) including recruited the tyrosine phosphatase SHP-1 and SHP-2 (Paul et al., 2000; Taylor et al., 1999) and resulted in down-regulated CD64-induced calcium influx (Paul et al., 2000; Ulyanova et al., 1999). Taken together, our findings may imply that CD33 could play an associate or even a crucial role in phagocytosis of microbial pathogens.

In conclusion, this study shows that the expression of CD33 on monocytes is influenced by various stimuli such as temperature as well as pathogen. Therefore, excessive processing

temperatures and the presence of *E. coli* should be taking into account when analysing leukocyte surface antigens. Further studies are required to elucidate the particular mechanisms of CD33 expression and its impact to the immune system.

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Figure legends

Figure 1. Schematic experimental design. Two sets of three tubes with whole blood from the test subject were handled as followed: two 50 µl aliquots of whole blood from ice bath (0 °C) cooler were either maintained always on ice or after pre-chilling warmed up to 37 °C. One 50 µl aliquot of whole blood from water bath (37 °C) was always prepared throughout at 37 °C.

Figure 2. Gating of the cells, CD14 and CD33 labeled cells. Three groups of cells were identified based on their forward scatter (FSC) and side scatter (SSC). By incubating these cells with Alexa Fluor® 488 anti-CD14 and PerCP/Cy5.5-anti-CD33, monocytes can be identified and relative median fluorescence intensity (RFI)was interpreted as positive for CD33 expression from the sample held at 0 °C.

Figure 3. Effect of incubation temperature and phagocytosis on CD33 expression on monocytes. Cells were chilled at 0 °C, warmed up from 0 °C to 37 °C, or kept throughout at 37 °C in the presence of *E. coli*. (A) The bar graphs show the percentage of relative median fluorescence intensity (RFI) of CD33 expression (n = 6). Three populations of monocytes were assessed for expression, cells chilled at 0 °C, those warmed from 0 °C to 37 °C, cells held throughout at 37 °C without stimulation (solid bar) or with *E. coli* stimulation (open bar). Each bar represents the mean ± SD, and asterisks indicate the significance of differences (* $p < 0.1$; **** $p < 0.0001$). (B) Flow cytometry analysis of CD33 fluorescence intensity (grey area, control; gravy line, monocytes chilled at 0 °C; black line, monocytes chilled at 0°C, warmed up from 0 °C to 37 °C; dash line, monocytes held through-out at 37 °C).

(C) Flow cytometry analysis of monocytes without staining, stained with two different clones CD33, and isotype control chilled at 0 °C, warmed up from 0 °C to 37 °C and held at 37 °C. No significant differences between the two clones WM53 and P67.6 were found.

Figure 4. Flow cytometry histograms of the phagocytosis activated by Fluorescein isothiocyanate (FITC)-labeled *E. coli* in the FITC-A channel of the monocyte (A) and granulocyte (B) population (grey area, chilled at 0 °C; black line, chilled at 0 °C, warmed up from 0 °C to 37 °C; dash line, held through-out at 37 °C)

Figure 5. Confocal laser scanning microscopy (CLSM) images of monocytes phagocytosed *E.coli* (A) Phagocytosis of Fluorescein isothiocyanate (FITC)-labeled *E. coli*, staining of the nucleus of monocytes with propidium iodide (fluorescence mode and overlay micrographs)
(B) The cells were stained at 0 °C with both Alexa Fluor® 488 anti- CD14 and PerCP/Cy5.5 anti-CD33 after performing phagocytosis of non-labeled *E. coli*.
(C)The cells were first incubated with both Alexa Fluor® 488 anti- CD14 and PerCP/Cy5.5 anti-CD33 for 30 min at 37 °C and then with non-labeled *E. coli* at 37 °C, which allowed to internalise antibody-bound CD14 and CD33. The monocytes phagocytosed *E. coli*, which results in intracellular fluorescence. Co-localisation of CD14 and CD33 staining was detected in yellow.

Figure 6. Flow cytometry histograms. (A) Opsonized *E. coli* (grey area) and opsonized *E. coli* incubated with PerCP-Cy5.5-anti-CD33 at 37 °C (black line). (B) Granulocytes and (C) monocytes in samples stained with PerCP-Cy5.5-anti-CD33 after performing phagocytosis (grey area), and samples incubated with PerCP-Cy5.5-anti-CD33 before stimulation with non-labelled *E. coli* at 37 °C (black line). The y-axis value varies depending on the number of cell count.

Table 1. Percentages of phagocytizing monocytes and granulocytes and mean fluorescence intensity upon Fluorescein isothiocyanate (FITC)-labeled *E. coli* treatment from healthy donors (n=6).

Fig. 4 Download full resolution image

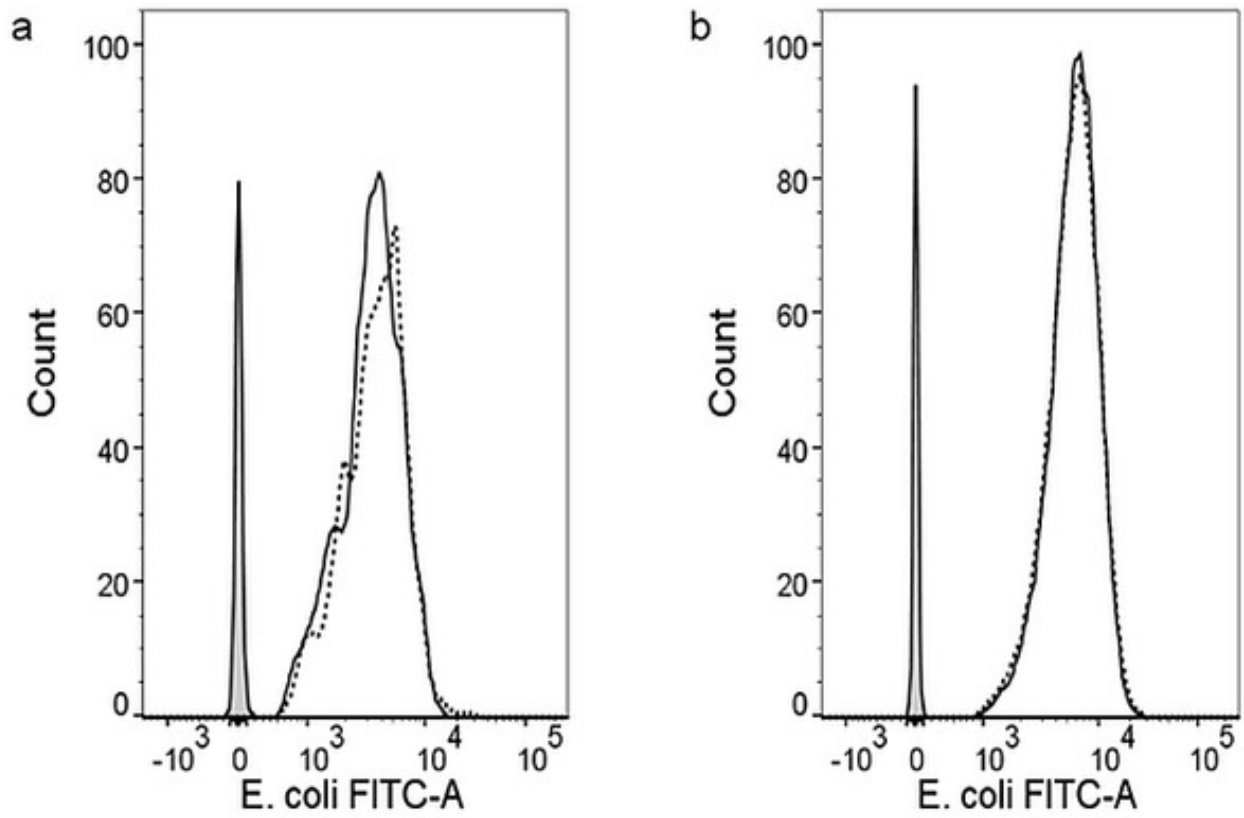
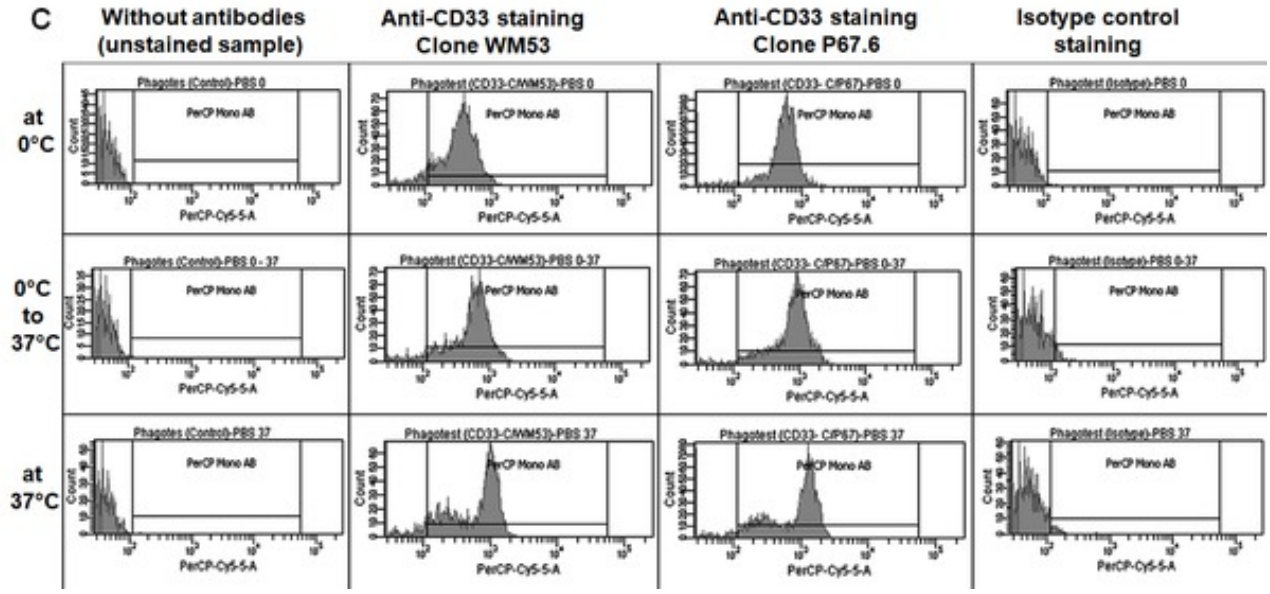


Fig. 3c [Download full resolution image]



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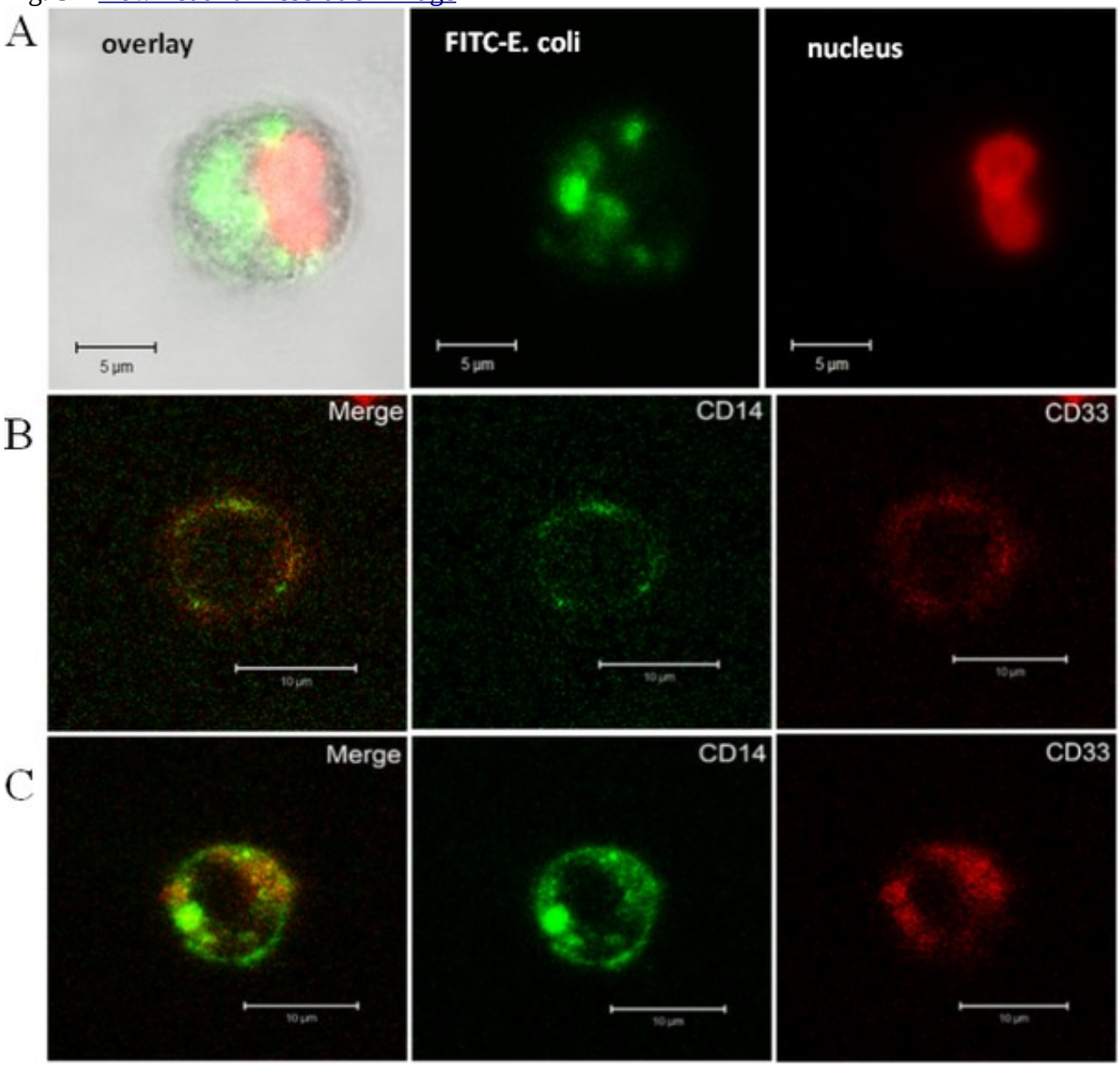

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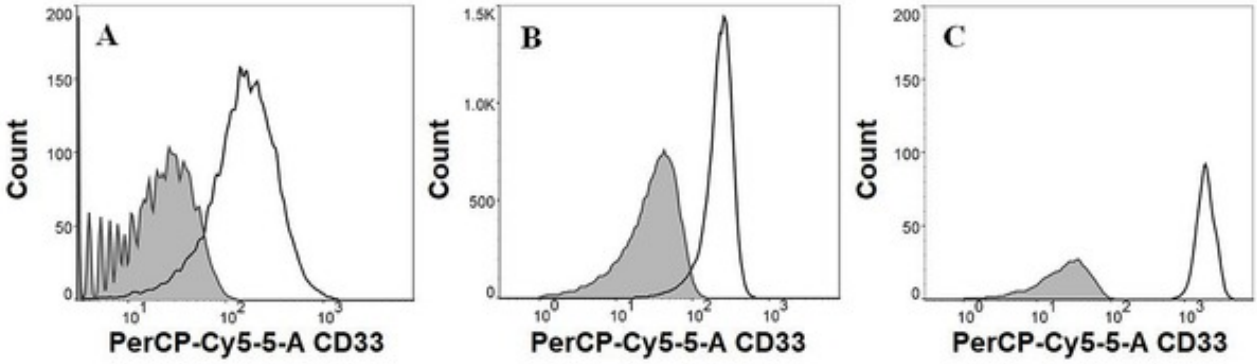
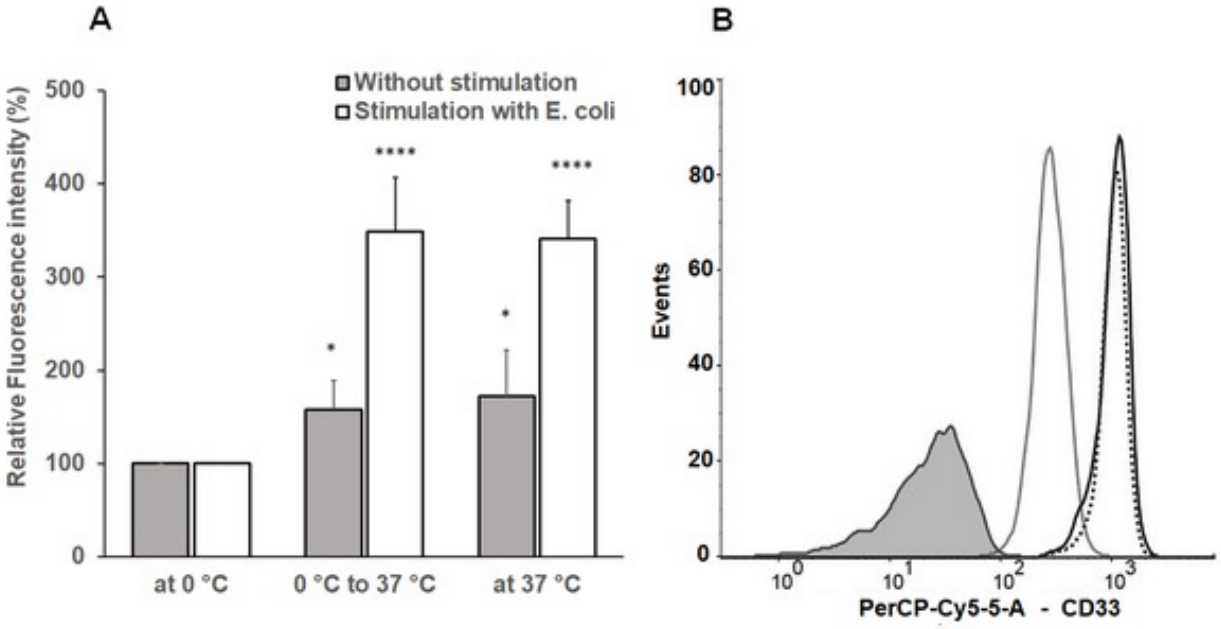


Fig. 3a+b [Download full resolution image]



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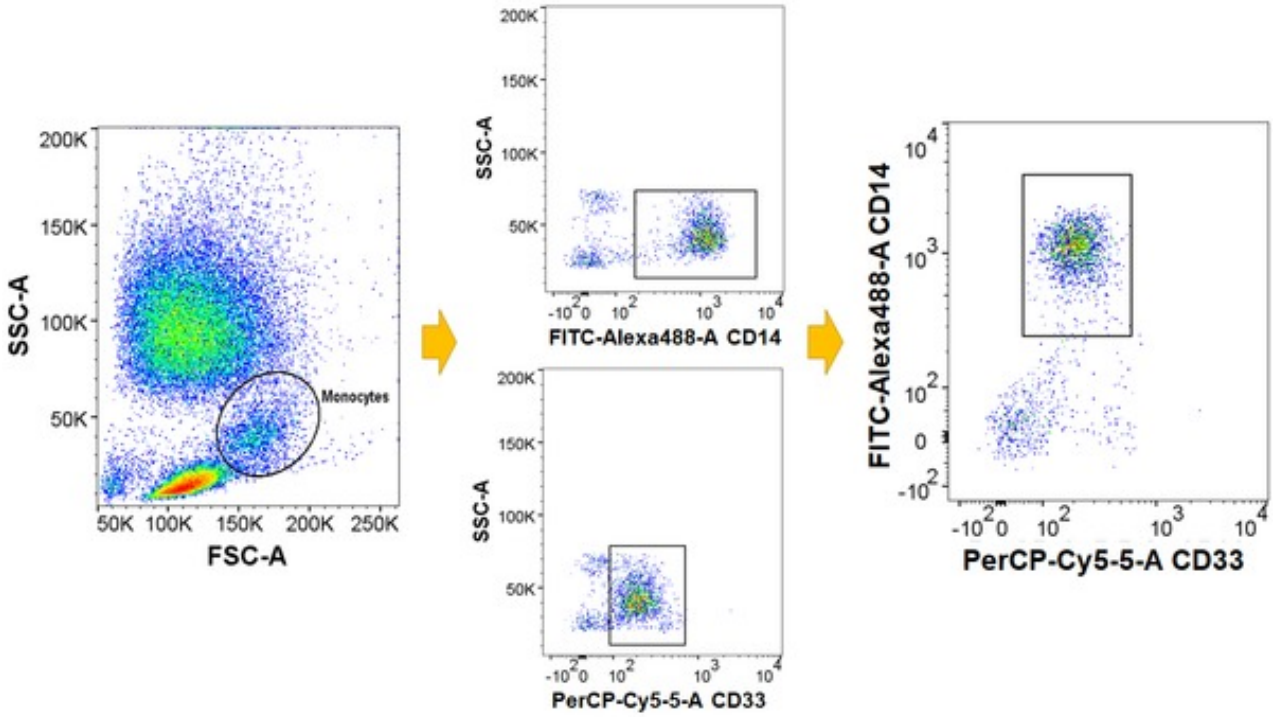

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