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TECHNICAL ADVANCE

New flow cytometry-based method for the assessment of the antibacterial effect of immune cells and subcellular particles

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Abstract

Techniques currently used for assessment of bacterial count or growth are time-consuming, offer low throughput, or they are complicated or expensive. The aim of the present work was to elaborate a new method that is able to detect the antibacterial effect of cells, subcellular particles, and soluble compounds in a fast, cost, and labor effective way. Our proposed technique is based on flow cytometry (FC) optimized for detection of small particles and on fluorescently labeled bacteria. It allows direct determination of the bacterial count in 3 hours. The effect of various human phagocytes and extracellular vesicles on gram-positive and gram-negative bacteria is investigated in parallel with the new, FC-based method, with colony counting and with our previous, OD-based method. Comparing the killing effect of wild type and NADPH oxidase-deficient murine neutrophils presents an example of detection of a clinically important deficiency. Strong correlation was obtained between the results of the different techniques, but the reproducibility of the FC-based test was superior to the OD-based test. The major advantages of the new technique are: rapidity, low cost, high throughput, and simplicity.

KEYWORDS

bacterial killing, extacellular vesicles, fluorescent staining, immunodeficiency, phagocytes

1 | INTRODUCTION

The major function of the immune system is to eliminate microorganisms from the host. Estimation of the antibacterial effect of immune cells or factors is important both in research and in the clinical field. However, fast and reliable measurement of antibacterial effect is still a challenging task. Many different methods have been elaborated and applied hitherto, but as summarized in Table 1, all have serious drawbacks and a simple, fast, and reliable technique is still lacking. Bacterial spreading and counting of CFU is regarded by many scientists as the gold standard, however it requires vast amount of manual labor and provides results only with a delay of 48 hours.

Flow cytometry (FC) is a powerful technique that is widely used for determination of various cellular functions and properties both in research and in clinical diagnostics, but its application in microbiological testing is underrepresented. The probable reason is that bacteria (approx. 0.5–1 μm) are significantly smaller than cells (5–20 μm) and this size is on the detection limit of most cytometers. Importantly, fluorescent labeling improves detectability and recent research on extracellular vesicles has initiated new approaches extending the detection limit of FC down to 200 nm.[34]

In this study, we present a new and simplified FC-based method to examine the antibacterial effect of any kind of cellular or subcellular samples. We compare our new FC-based method to CFU counting and to the OD-based assay developed and validated earlier in our laboratory.[8] By devising the present test, the main goals were reliability, high throughput, low cost, low waste production, and results in short time.

2 | MATERIALS AND METHODS

2.1 | Materials

HBSS with calcium and magnesium but without glucose was from GE Healthcare Life Sciences (South Logan, UT, USA), Saponin was from Calbiochem-Novabiochem Corporation (San Diego, CA, USA), Zymosan A from Sigma Aldrich (St. Louis, MO, USA), Ficoll-Paque and Percoll from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). The lysogeny broth (LB medium) contained 10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L distilled water, pH was adjusted to 7.0. Acridine Orange

Abbreviations: AO, acridine orange; EV, extracellular vesicles; FC, flow cytometry; LB, lysogeny broth; NOX, neutrophil oxidase; PMN, polymorphonuclear cell

TABLE 1 Existing methods to measure antibacterial activity of immune cells or subcellular units

	Used	Measured parameter	Tested bacteria	Tested sample	Labor intensity	Digital processing	Throughput	Expert needs	Contaminated waste	Costs	References
Plate CFU counting	+++	Bacteria count	All kind	Cellular Subcellular	++	minimal	low	no	+++	+	1–7
OD changes based methods	++	**Bacteria growth**	All kind	Cellular Subcellular	+	++	medium	++	++	+	8–14
Enzyme reaction based methods	++	Enzyme activity (formazan formation)	**Genetically modif.**/all	Cellular Subcellular Lysis dependent	+	+	medium	+	+	++	15–20
PCR based method	+	Gene amount	All kind	Cellular Subcellular	+	++	medium	+	+	+++	21,22
Fluorescence quenching based techniques	+	Bacteria count	**Endogen fluorescent**	**Only cells**	+	+	high	+++	+	++	23–25
FC based methods	+	**Membrane potential or integrity**	All kind	Cellular Subcellular	+	+	high	+++	+	+++	26–33
Present method	-	*Bacteria count*	*All kind*	*Cellular Subcellular*	+	+	*high*	++	+	+	

Fields in bold emphasize the weak points of the method.

was from Serva-Feinbiochemica (Heidelberg, Germany). All other used reagents were of research grade. Methicillin-sensitive *S. aureus* (ATCC: 29213) and *E. coli* (ATCC: ML-35) were used. GFP-expressing and chloramphenicol resistant *S. aureus* (USA300) was a kind gift of Professor William Nauseef (University of Iowa).

2.2 | Preparation of human polymorphonuclear cells (PMN), monocytes, and erythrocytes

Venous blood was drawn from healthy human adult volunteers according to procedures approved by the National Ethical Committee (ETT-TUKEB No. BPR/021/01563-2/2015). Red blood cells were separated from the white blood cells by dextran sedimentation. Thereafter PMN were separated from the monocyte-lymphocyte fraction by 62.5% v/v Ficoll gradient centrifugation (700 *g*, 40 min, 22°C). Remaining red blood cell contamination was removed by hypotonic treatment. The obtained sample contained 95% or more PMN. Monocytes were separated from the other leukocytes by 46% v/v Percoll gradient centrifugation (700 *g*, 40 min, 22°C). The obtained sediment contained mostly monocytes and some contaminant lymphocytes but no neutrophils. All cell types were used in 10^7 cells/mL concentration.

2.3 | Mice and isolation of murine PMN

Neutrophil oxidase 2 (NOX2)-deficient animals on C57Bl/6J background[35] were a kind gift from Professor Miklós Geiszt (Semmelweis University, Budapest). All NOX2-deficient mice were males (NOX2$^{-/0}$) 11–14 week old. Age- and sex-matched C57Bl/6J animals were used as controls. Genotyping was performed by allele-specific PCR.
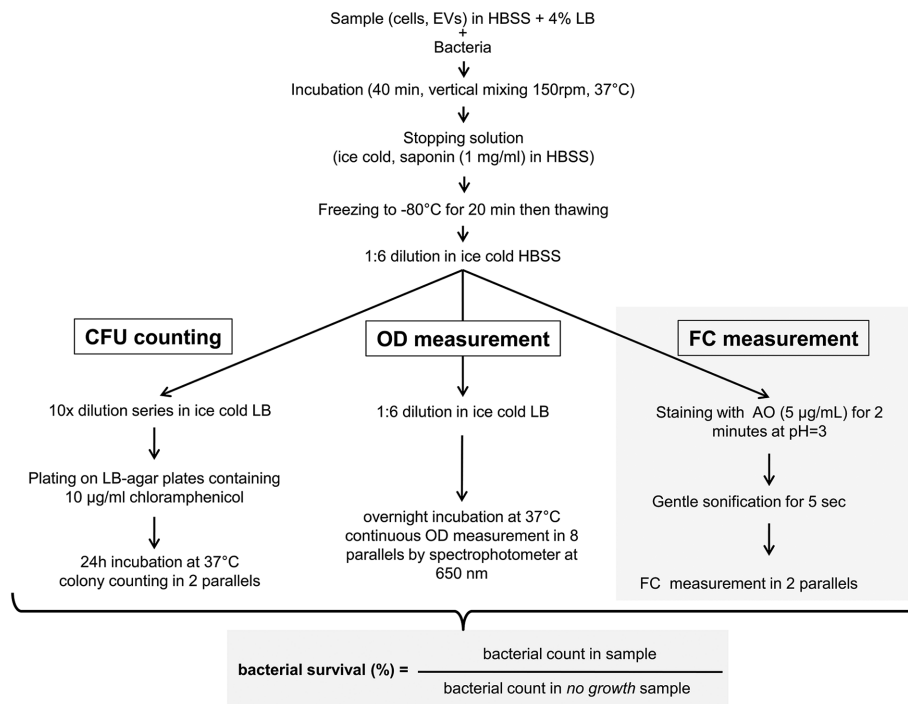
Murine neutrophils were isolated from the bone marrow of the femurs and tibias of mice by hypotonic lysis followed by Percoll (GE Healthcare) gradient centrifugation as previously described.[36] Cells were kept at room temperature in Ca^{2+}- and Mg^{2+}-free medium until use (usually less than 30 min) and prewarmed to 37°C before activation. Neutrophil assays were performed at 37°C in HBSS supplemented with 20 mM Hepes, pH 7.4.

2.4 | Opsonization of Zymozan A and bacteria

Zymosan A (5 mg) or 1 mL bacteria ($OD_{600} = 1.0$) were opsonized either with 200 μL pooled human serum or with 800 μL pooled murine serum for 20 min at 37°C. Opsonized particles were centrifuged (10,000 *g*, Hermle Z216MK 45° fixed angle rotor, 10 min, 4°C), and washed, then resuspended in 1 mL HBSS.

2.5 | Preparation of extracellular vesicles (EV) from human PMN

PMN were activated with opsonized Zymosan A particles for 20 min at 37°C in linear shaking water bath with 80 rpm. After activation, cells were sedimented (500 *g*, Hermle Z216MK 45° fixed angle rotor, 5 min, 4°C). Upper 500 μL of the supernatant was filtered through a 5 μm pore sterile filter (Sterile Millex Filter Unit, Millipore,

Sample (cells, EVs) in HBSS + 4% LB
+
Bacteria
↓
Incubation (40 min, vertical mixing 150rpm, 37°C)
↓
Stopping solution
(ice cold, saponin (1 mg/ml) in HBSS)
↓
Freezing to -80°C for 20 min then thawing
↓
1:6 dilution in ice cold HBSS

CFU counting	**OD measurement**	**FC measurement**
10x dilution series in ice cold LB	1:6 dilution in ice cold LB	Staining with AO (5 µg/mL) for 2 minutes at pH=3
↓	↓	↓
Plating on LB-agar plates containing 10 µg/ml chloramphenicol		Gentle sonication for 5 sec
↓	overnight incubation at 37°C continuous OD measurement in 8 parallels by spectrophotometer at 650 nm	↓
24h incubation at 37°C colony counting in 2 parallels		FC measurement in 2 parallels

$$\text{bacterial survival (\%)} = \frac{\text{bacterial count in sample}}{\text{bacterial count in } no\ growth \text{ sample}}$$

FIGURE 1 Experimental scheme of CFU counting, the OD and the FC based methods

Billerica, MA, USA). The filtered fraction was sedimented (15,700 g, Hermle Z216MK 45° fixed angle rotor, 5 min, 4°C), the pellet was carefully resuspended in 500 μL HBSS, followed by addition of 20 μL LB to each sample. To examine the dose-dependency of EV on bacterial survival, a dilution series of the EV fractions was prepared. Corresponding to our earlier experiments,[14] EV derived from 10^7 PMN was taken as reference (1 EV). Compared to this, we prepared diluted (0.1 EV and 0.5 EV) and more concentrated (5 EV) samples. As a negative control we applied heat-inactivated (20 min, 100°C, orbital shaking 600 rpm) 1 EV.

2.6 | Measurement of bacterial survival

Opsonized bacteria (2.5×10^7) were added to 5×10^6 human cells or to 500 μL EV suspended in HBSS containing 4% v/v LB or to 5×10^6 murine PMN (in 500 μL HBSS) (Fig. 1). During a 40 min coincubation step at 37°C, the bacterial count decreases or increases depending on the samples' antibacterial effect and the growth of bacteria. At the end of the incubation, 2 mL ice-cold stopping solution (1 mg/mL saponin in HBSS) was added to stop the incubation and lyse cells or EV. After a freezing step at −80°C for 20 min, samples were thawed to room temperature. Complete release of surviving bacteria from the phagocytes was controlled both by flow cytometry and fluorescent microscopy (data not shown). Thus, our test examines true bacterial survival and not phagocytosis. The initial bacterial count was determined by adding the same quantity of bacteria to an extra aliquot of HBSS containing 4% v/v LB at the end of the 40 min incubation, immediately before addition of the ice-cold stopping solution. We termed this sample as "no growth" sample. Quantification of the samples was performed in parallel using the CFU counting, the OD and the FC-based method. Finally, the bacterial survival was expressed in percentage as the ratio

of the bacterial count determined in the 40 min and in the "no growth" samples (Fig. 1).

2.7 | CFU counting method

Bacterial suspensions were serially diluted and plated on LB-agar in two parallels to enumerate surviving bacteria. To minimize the imprecision due to bacterial contamination, agar plates contained 10 μg/mL chloramphenicol to ensure selective *USA300* up-growth.

2.8 | Spectrophotometry-based quantification of bacterial count

Bacterial growth was followed as changes in OD using a shaking microplate reader (Labsystems iEMS Reader MF, Thermo Scientific) for 8 hours, at 37°C, at 650 nm (Fig. 1). After the end of this growth phase the initial bacterial counts were calculated indirectly using an equation similar to PCR calculation, as described previously.[8]

2.9 | Flow cytometry-based quantification of bacterial count

Measurements were executed by a BD FACSCalibur flow cytometer, optimized for small particle detection. As both the size and the refractive index of EV and bacteria are very similar, the gating procedure was similar to the EV detection and quantitation used before in.[13] Briefly, HBSS was used for setting the thresholds to eliminate instrument noise (Figs. 2A and B). The upper size limit of bacterial detection range was set by 3.8 μm fluorescent beads (SPHERO Rainbow Alignment Particles from Spherotech Inc., USA). The lower size limit of bacterial detection was set to exclude the
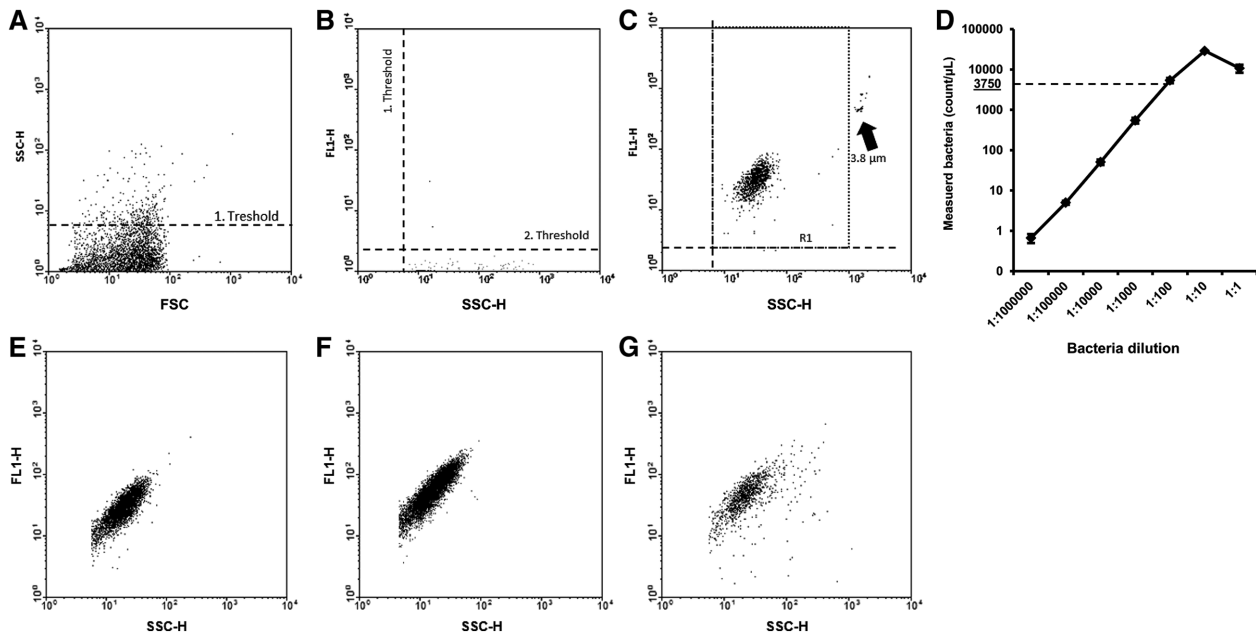
FIGURE 2 Gating strategy of flow cytometric measurements. In panel A side scatter is presented against forward scatter, whereas in panels B, C and E–G, green fluorescence is presented against side scatter. (A and B) Representative dot plots on pure HBSS medium to set thresholds. (C) Representative dot plot on fluorescent beads (3.8 μm) and GFP-expressing *S. aureus* (diameter ca. 0.8 μm) measured in parallel to set R1 gate; (D) Calibration of the optimal flow rate range of the BD FACScalibur flow cytometer. *USA300* bacteria were used for calibration. The undiluted sample had an optical density of 1.00 at 600 nm. Bars represent $\pm$SEM; $n = 5$ (E). Representative dot plots on *USA300* bacteria population at start of killing step, (F) at the end of 40 min incubation in HBSS + 4% LB, and (G) at the end of 40 min killing in HBSS + 4% LB with human PMN

instrumental noise, thus detected particles were no smaller than 300 nm[34] (Fig. 2C). Since the bacteria's size range (around 500–1000 nm) is near to the detection limit of a conventional flow cytometer, fluorescent labeling was used to improve FC detection of particles. For fluorescent labeling of nonfluorescent bacteria Acridine Orange (N,N,N′,N′-Tetramethylacridine-3,6-diamine) was used in 5 μg/mL final concentration for 5 min at room temperature at pH = 3. Samples were very gently sonicated (Bandelin Sonopuls HD 2070, 10% power) for 5 s to disrupt bacterial clumps and doublets. Our control measurements agreed with previous findings[37] and indicated that weak sonication did not interfere with bacterial viability and acridine orange (AO) staining (data not shown). The fluorescence gate was set above the endogenous fluorescence of nonlabeled bacteria detected by the "green" fluorescence detector (530/30 nm). Bacteria were enumerated in the R1 gate (Fig. 2C). To stay in the reliable detection range of the cytometer and to avoid swarm detection an optimal flow range was defined with a 10-fold dilution scale of fluorescent bacteria. The flow rate was held during measurements under 1000 events/s (3750 events/μL, dashed line in Fig. 2D). Furthermore, all samples were measured again after a 2-fold dilution to control linearity of measurements. FC data were analyzed with Flowing 2.5 Software (Turku Centre for Biotechnology, Finland).

2.10 | Statistics

Statistical analysis was performed using GraphPad Prism 6 for Windows (La Jolla, California, USA) and P-values < 0.05 were considered as significant.

3 | RESULTS AND DISCUSSION

3.1 | Bacterial survival in different solutions with or without neutrophils

Antibacterial assays are usually carried out in media not supporting bacterial growth such as PBS or HBSS. Incubation in such media provides the initial bacterial count, but these tests only allow detection of bactericidal but not bacteriostatic effects. In our antibacterial assay initial bacterial count was defined by the "no growth" sample (marked as 100% bacterial survival) and HBSS + 4% LB was used as general assay solution. The diluted broth ensures moderate growth that can be inhibited by bacteriostatic effects. Samples containing bacteria under different conditions were divided and bacterial count was determined in parallel by two or three independent methods.

As shown in Fig. 3A, incubation of bacteria for 40 min in pure HBSS had no effect, whereas incubation in HBSS + 4% LB resulted in almost doubling of the bacterial count. Next neutrophilic granulocytes (PMN) were coincubated so that the cell to bacteria ratio was altered hundredfold. Under all conditions bacterial count was significantly diminished by the end of 20 or 40 min. Analysis of the results obtained by CFU counting compared to the OD and the FC-based methods is presented in Fig. 3B. Very strong correlation was obtained between CFU counting and both other tested methods.

In the following experiments, results of the new FC-based method were only compared to the OD-based method that offers higher throughput and requires less manual labor. First, we tested our assay with different bacterial strains. In case of the GFP-expressing *S. aureus*
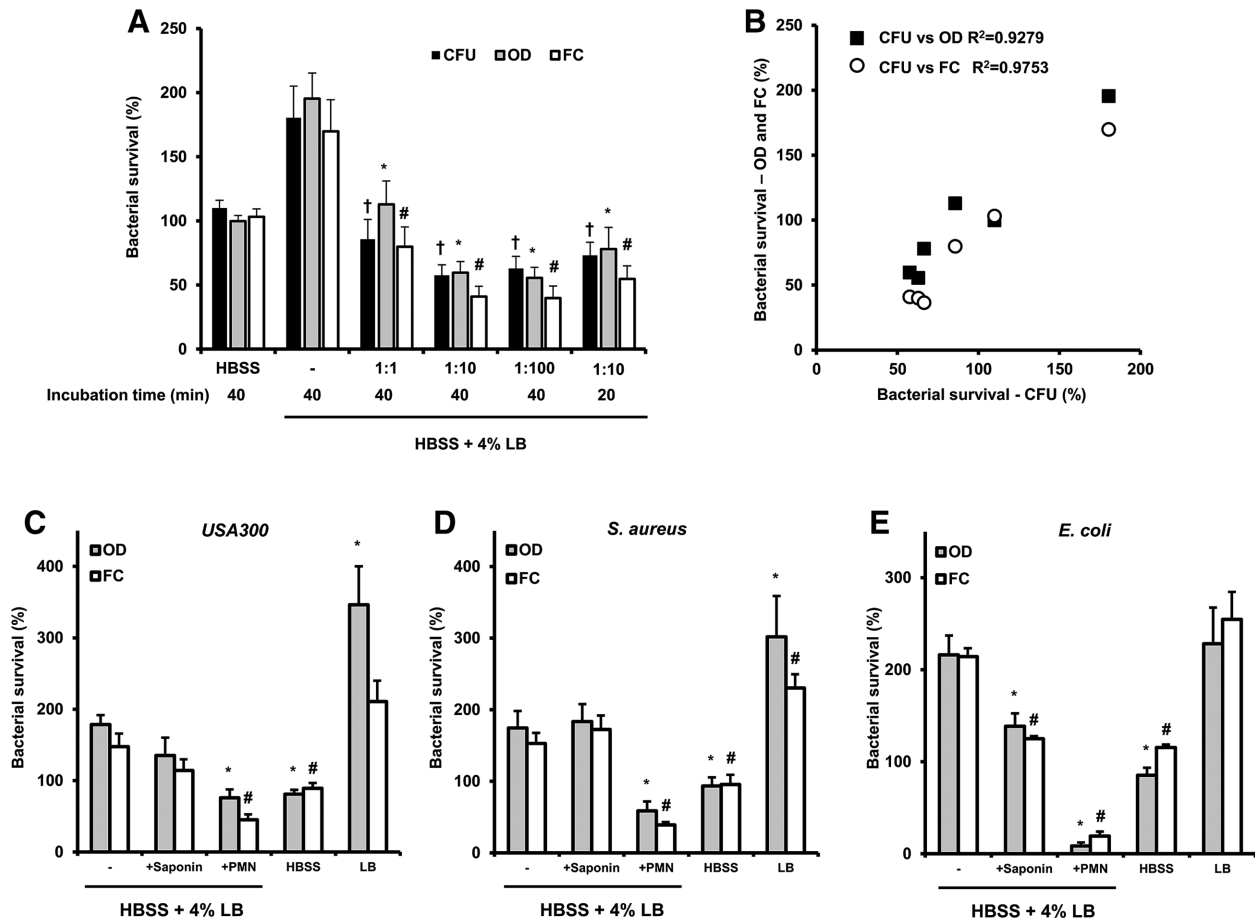
FIGURE 3 Comparison of the FC-based method with previously applied techniques for measuring bacterial survival in different circumstances. (A) Survival of GFP expressing *S. aureus (USA300)* at different PMN to bacteria ratio and killing time. (B) Correlations of OD and FC-based methods to CFU counting. (C–E) Survival in different media with or without PMN present of *USA300* (C), AO stained *S. aureus* strain (D), and AO stained *E. coli* strain (E). Black, gray, and white bars represent results obtained by the CFU counting, OD-based and FC-based method, resp.; ($n = 6$; mean + SEM; Significance was calculated in relation to the assay solution HBSS + 4% LB, †, *, and # represent $P < 0.05$; two sample Student's t-test)

strain, *USA300* moderate but clear growth was detected in HBSS + 4% LB with both techniques (Fig. 3C). The weak detergent saponin, used in our assay to release viable bacteria from PMN, did not significantly interfere with the growth of the GFP-expressing *S. aureus* strain (Fig. 3C). The significant antibacterial effect of neutrophils (PMN/bacteria = 1:10) was evident in both assays. As methodological reference points, bacterial survival was also tested in HBSS (no growth) and in LB (maximal growth). In HBSS the expected 100% survival rate was detected and the calculated SD of measurements was very low in both assays. In contrast, significant difference was observed between the compared two techniques when bacteria were grown in LB (Fig. 3C; see later in details).

We carried out the same experiments with two other bacterial strains that did not express endogenous fluorescent protein. These bacteria were stained with AO for the FC measurement. Wild type *S. aureus* showed the same survival pattern as the GFP-expressing strain (Fig. 3D). The gram-negative *E. coli* was moderately affected by the detergent saponin and more effectively eliminated by PMN than *S. aureus* strains (Fig. 3E). These differences between *S. aureus* and *E. coli* fit to previous findings on different sensitivity of the two strains to PMN.[8] Taken together we show that the test works equally well for

gram negative and gram-positive bacteria and with bacteria expressing fluorescent protein or stained with a simple fluorescent dye.

3.2 | Antibacterial effect of different blood cells

To test the wider applicability of the new FC-based method, the antibacterial effect of other blood cells was also investigated. Compared to the control, both PMN and monocytes decreased the survival of *S. aureus* in contrast to erythrocytes that had no antibacterial effect, demonstrating that inert cellular material itself does not modify or falsify the measured result (Fig. 4A).

In order to test a clinically relevant alteration of killing ability, we investigated neutrophils from wild type and NADPH oxidase (NOX2) knockout mice deficient in the membrane-localized subunit of the enzyme.[35] Both methods showed significant difference in bacterial killing between wild type and NOX2 knock-out murine PMN after a 40 min test (Fig. 4B). Specificity and sensitivity of the two methods were calculated from the data presented in Fig. 4B and proved to be very similar. Killing was regarded effective when bacterial survival was smaller than the lower 95% confidence limit of bacterial survival in HBSS (90.31%). Specificity was 81% for the OD-based technique
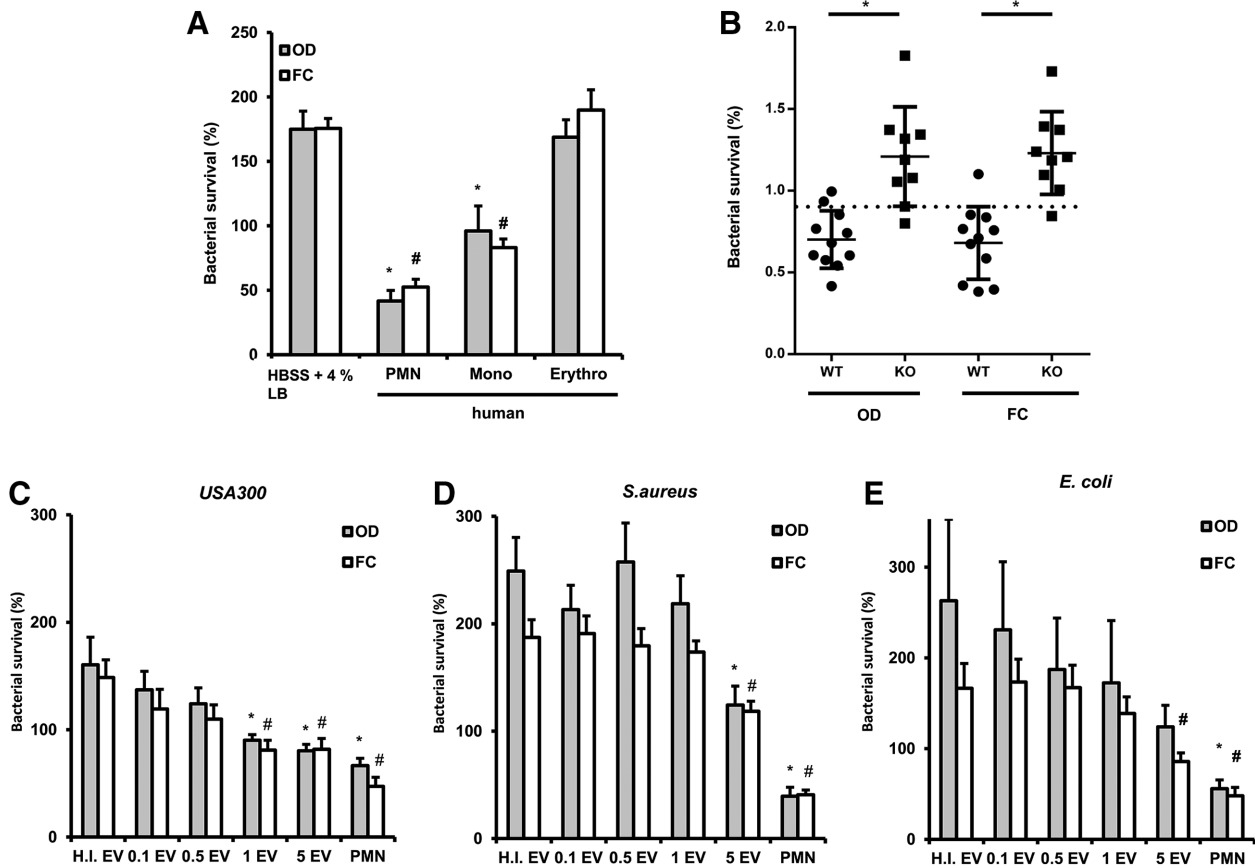
FIGURE 4 **Testing of the antibacterial effect of different blood cells and subcellular particles.** Gray and white bars represent results obtained by the OD-based and FC-based method. (A) Effect of human blood cells on AO stained *S. aureus*. ($n = 7$ or 6 (in case of erythrocyte), mean + SEM, * and # represent $P < 0.05$ in relation to HBSS + 4%, two sample Student's *t*-test). (B) Antibacterial effect of 11–14-week-old male murine wild type (C57Bl/6J) and NOX2 KO (NOX2$^{-/0}$) PMN on GFP expressing *S. aureus (USA300)*. ($n = 11$ (WT), and 9 (KO) mean + SEM, * represents $P < 0.05$, two sample Student's *t*-test). The dotted line represents the lower 95% confidence limit of bacterial survival in HBSS (90.31%). (C–E) Dose-dependent antibacterial effect of PMN-derived EV: (C) Survival of *USA300* ($n = 8$) (D) AO stained *S. aureus* strain ($n = 6$), and (E) AO stained *E. coli* strain ($n = 6$); mean + SEM, * and # represent $P < 0.05$ in relation to heat inactivated EV (H.I. EV)

and 91% for the FC-based technique. Sensitivity was identical for both techniques (89%). The present findings are in agreement with previous observations on superoxide-dependent elimination of *S. aureus* by Rada et al.[8] and lack of superoxide production in NOX2-deficient neutrophils.[35]

3.3 | Dose-dependent effect of PMN-derived extracellular vesicle (EV) on bacteria

Next, we asked the question whether our new FC-based method was suitable for detection of the antibacterial effect of subcellular particles. As we and others showed before, activated PMN produce EV that impair the growth of both gram positive and negative bacteria.[14,38,39] However, dose dependency of EV-mediated antibacterial effect was not showed before. In our previous experiments, we tested the antibacterial effect of EV produced by 1×10^7 activated PMN (referred to as "1 EV"). In the current experiments, we tested a broader range from tenfold diluted to fivefold concentrated EV preparations on all three bacterial strains (Fig. 4C–E). The antibacterial effect was evident with all three tested bacteria, but their sensitivity proved to be different. When testing the *USA300* strain statistically significant

effect was attained at "1 EV" concentration. In case of the two other bacterial strains, statistically significant antibacterial action was only brought about by fivefold concentrated EV preparation.

3.4 | Reproducibility of the results

To examine the reproducibility of our new FC-based assay we compared survival of *S. aureus* in HBSS + 4% LB in the presence and absence of PMN as determined in five separate experimental series (in each series 6 or 8 identical experiments were carried out within one month) (Fig. 5A). PMN effectively reduced bacterial survival in all five experimental series detected by either technique, although the FC-based method seemed to produce more homogenous results. In the absence of PMN very similar bacterial growth was detected by both techniques, although the deviation was smaller in case of the FC-based method (Fig. 5A).

In Fig. 5B the result of every single experiment of the five series is presented by one dot. The mean of the total of 32 experiments carried out with the two different methods fits very well but the deviation of individual data is significantly lower in case of the FC-based method.
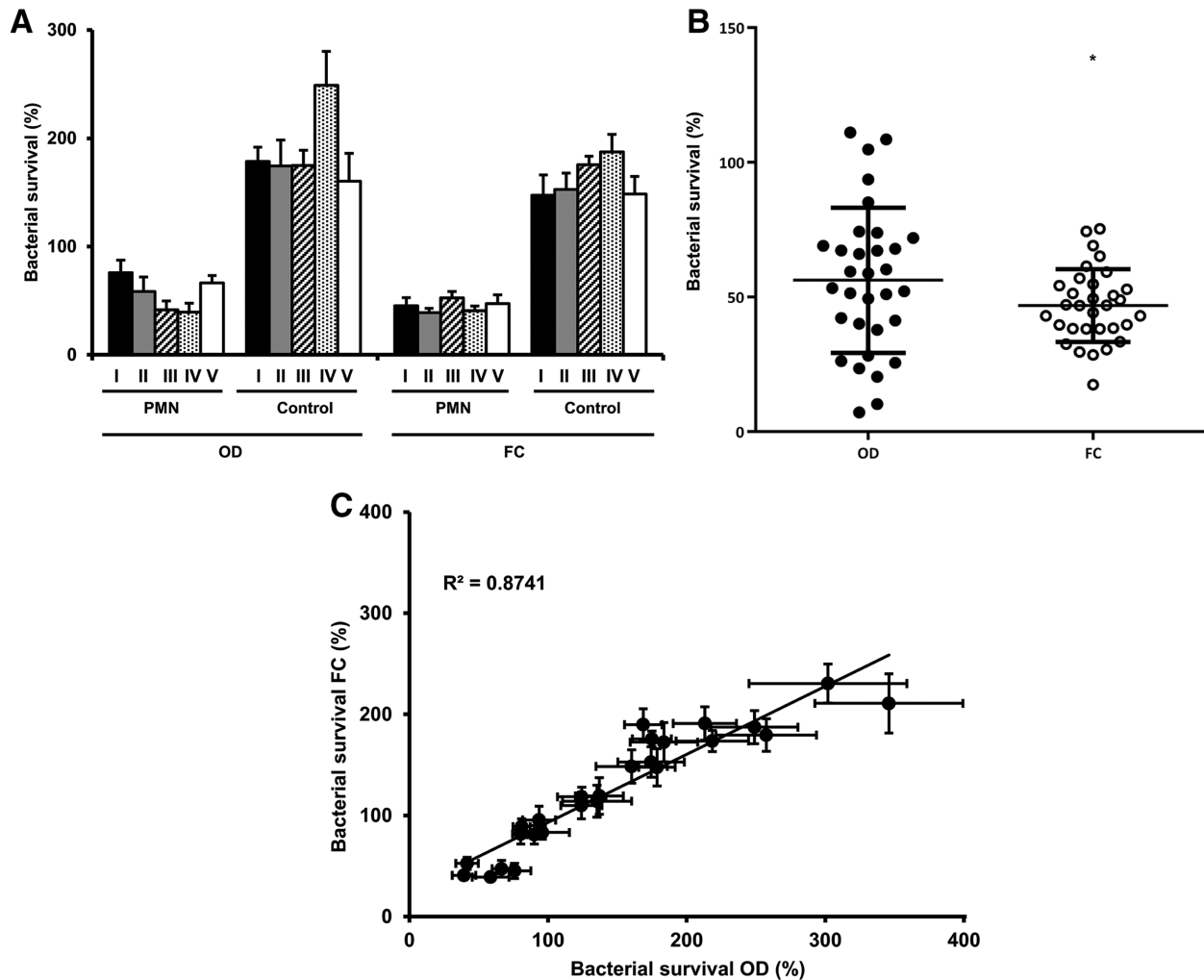
FIGURE 5 **Comparison of the reproducibility of the FC-based and OD-based methods**. (A) Bacterial survival of *S. aureus* in five separate experimental series with or without PMN present (test medium was HBSS + 4% LB in all cases). In each series 6–8 experiments were carried out (mean + SEM). (B) Scattering of every single measurement of *S. aureus* survival in the presence of PMN ($n = 32$, mean $\pm$ SD). *F*-test showed significant difference in variances of the two methods (signed as [*]); however, *t*-test with Welch's correction showed nonsignificant difference in means. (C) Summarized linear regression of bacterial survival measured by OD- and FC-based methods ($n = 37$, $\pm$SEM)

Figure 5C summarizes all the data of *S. aureus* survival presented in Figs. 3–5. The high value of the linear regression coefficient indicates good comparability of the two methods up to approx. 300% growth rate. At very high growth rate, incubation of bacteria for 40 min in nutrient rich medium resulted in an active reproductive state compared to the quiescent "no growth" reference sample. This difference significantly influenced the growing up time in the OD-based method and led to overestimation of the initial bacterial count.

4 | CONCLUSION

In our opinion, our new method offers advantages over all techniques summarized in Table 1. However, the most important advantage is in the time factor. Applying the new FC-based method, bacterial count of the sample can be determined in 3 hours as compared to 16 hours with the OD-based test and 48 hours of CFU counting. In cases of symptomatic immunodeficiencies of unknown origin (congenital or acquired), investigation of phagocytic functions, including bacterial killing is recommended.[40] In these cases, the time difference in obtaining diagnostic results is critical. The proposed assay determines the number of bacteria, thus no conversion or further processing of the measured data is required. Depending on the property of the used medium, i.e. whether it allows bacterial growth or not, both bacteriostatic and bactericidal effects can be measured.

Taken together, we propose a new, fast, easy, reliable, and reproducible FC-based test that is suitable for high throughput quantitation of bacteriostatic or bactericidal effect of any immune cell or noncellular material and provides large potential both for research and for clinical purposes.

DISCLOSURE

The authors have no conflict of interest to declare.

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