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RESEARCH ARTICLE

Flow Cytometry Assessment of Bacterial and Yeast Induced Oxidative Burst in Peripheral Blood Phagocytes

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Objective: The aim of this study was to verify in our laboratory conditions the performance criteria of a commercial kit (Phagoburst™, Glycotope Biotechnology) as described by the producers. We have also partially altered the use of the available kit by introducing a non-opsonized *Candida albicans* stimulus, in addition to the opsonized *Escherichia coli* stimulus provided by the manufacturer. **Material and methods**: The peripheral blood samples of 6 clinically healthy adults were tested in triplicate according to the manufacturer recommendations. The intra-assay imprecision as well as the ranges of neutrophil and monocyte burst activation triggered by various stimuli were assessed. **Results**: The activation range of granulocytes and monocytes was similar to the one described by the producer in the presence of *E. coli* (granulocytes: 78.45-99.43% versus 99.6-99.95%, average %CV of 1.53% versus 0.1%, monocytes: 54.63-92.33% versus 81.80-96.67, average %CV 6.92% versus 1.1%). The leukocyte range of activation in the presence of non-opsonized *C. albicans* was comparable to the one triggered by the fMLP (N-formyl-methionyl-leucyl-phenylalanine) stimulus. **Conclusion**: The intra-assay precision obtained in our laboratory conditions, as well as the ranges of activated leukocytes, are comparable to the ones described by the producer when using *E. coli* as a stimulus. The present study shows that introducing an extra fungal stimulus for burst oxidation assessment could provide additional information regarding the non-specific cellular immune response, particularly in patients at risk for candidemia.

Keywords: flow cytometry, method verification, intra-assay precision, reactive oxygen species, fungal bloodstream infection

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Introduction

The peripheral blood phagocytes (PBP) have long been acknowledged as key factors in bacterial and fungal infections. As first line of defense, they phagocyte and kill microorganisms through a combination of mechanisms that include the production of reactive oxygen species (ROS) [1].

Along the years, flow cytometry methods have been developed in order to quantify the production of ROS using bacteria as stimulus [2,3]. Different authors have described methods for burst oxidation assessment in peripheral blood mononuclear cells (PBMC) using isolated leukocytes and different strains of *Candida* as stimuli as well as different incubation periods [4-8]. Recent years have seen the development of several commercial kits that use whole blood, not isolated PBMC as many methods used before, and unlabeled opsonized *Escherichia coli* as stimulus for PBP activation besides other chemical stimulants [9]. Up to the present moment none of the available commercial kits used fungi as stimulus.

The aim of the present study was to verify in our laboratory conditions the performance criteria of the commercial kit Phagoburst ™ (Glycotope Biotechnology) as recommended by the producers. We have also partially altered the use of the available kit by introducing a non-opsonized *Candida albicans* suspension for testing as stimulus with the purpose to assess the cellular immune response in fungal bloodstream infections.

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Materials and methods

We conducted a study that aimed to verify the performance parameters of the *Phagoburst ™ (Glycotope, Biotechnology)* commercial kit in our laboratory conditions and to validate as well the use of a new stimulus – a non-opsonized *C. albicans* suspension in order to verify the PBP innate immune response to fungal stimuli.

The study was conducted according to the World Medical Association Declaration of Helsinky and was approved by the Ethics Committee of the Emergency Clinical County Hospital of Tîrgu Mureş, No.19204/29th of September 2014 as well as the Ethics Committee of the University of Medicine and Pharmacy of Tîrgu Mureş, No.53/22nd of April 2015. Informed consent was given by each enrolled adult.

Blood samples

Whole venous blood specimens were collected from 6 healthy adults on BD sodium heparin tubes as recommended by the producers of the *Phagoburst ™* (Glycotope Biotechnology) commercial kit. The volunteers were chosen by absence of infectious history and clinical signs of infection. All samples were tested in triplicate within one hour after collection, being kept meanwhile on a covered ice bath.

Assay for the evaluation of cell burst activity

Phagoburst ™ was created to investigate the altered oxidative burst activity present in various pathologies and to evaluate the effects of drugs. It allows quantitative assess-

ment of PBP oxidative burst. The kit contains an unlabeled opsonized *E. coli* bacteria as particulate stimulus, the protein kinase C ligand phorbol 12-myristate 13-acetate (PMA) as high stimulus, the chemotactic peptide N-formyl-MetLeuPhe (fMLP) as low physiological stimulus, dihydrorhodamine 123 (DHR 123) as a fluorogenic substrate and necessary reagents.

In order to quantify the production of ROS by PBP that could be triggered by a fungal pathogen in neonates which are known to have low opsonin concentration and degree of activation [10], we introduced an extra fungal stimulus for testing in the form of a *C. albicans* yeast suspension. The reference strain *C. albicans ATCC 10321* was cultured aerobically on Sabouraud chloramphenicol agar for 18 hours at 35°C. An 0.5 optical density inoculum of *C. albicans* (approximate cell concentration of 1-5 x 10^6 colony forming units/mL) was prepared for each testing round by Using Vitek2 Densichek densitometer (Biomerieux, France). The yeast suspension was not exposed to any supplementary opsonins then those present in the 100 µL whole blood used for testing. The yeast cells underwent no inactivation prior to exposure to whole blood. May Grunwald Giemsa stained blood smears performed after a 10 minutes period of incubation of whole blood with the yeast stimulus showed no budding, pseudohyphae or hyphal growth.

Heparinized whole blood (100 µL) within 1 hour after sampling was incubated with 20 µl of each of the above mentioned stimuli for 10 minutes at 37°C on a water bath. The ROS produced during the oxidative burst by the phagocytes was monitored by oxidation of 20 µl DHR 123 which served as an oxidative fluorogenic substrate. A volume of 2 ml lysing solution which removed the erythrocytes and resulted in a partial fixation of the leukocytes was added to stop the burst oxidation reaction. After centrifugation and one washing step, 200 µL DNA staining solution was added to exclude aggregation artifacts of bacteria, fungi or cells. The DNA staining required 10 minutes incubation at 0°C, protected from light. Samples were thus ready for FACS analysis that was performed within 30 minutes following DNA staining.

Flow cytometry analysis

Each blood sample was tested in triplicate. Cells were analyzed by flow cytometry using a 488 nm argon-ion excitation laser. As recommended by the producer, during data acquisition a "live gate" was set in the red fluorescence histogram on the events that had at least the same DNA content as a human diploid cell with the purpose of precluding bacteria or fungi aggregates that had the same scatter light properties as the leukocytes from analysis. An average number of 15000 leukocytes per sample were collected.

The percentage of cells that produced ROS (recruitment) as well as their mean fluorescence intensity (MFI) (activity, amount of cleaved substrate) was quantified. The relevant leukocyte cluster was gated in the software program in the scatter diagram (linear FSC vs linear SSC) and its rhodamine 123 green fluorescence was collected in the FL1 channel (standard FITC filter set) and analyzed. A negative control sample without any stimulus was always run as a negative background control to set a marker for fluorescence (FL1) so that less than 3% of the events were positive. The percentage of activated cells in the test samples was then set by counting the number of events above this threshold. The mean fluorescence correlates with oxidation quantity per individual leukocyte.

Data collection and analysis

Results for every round of tests of each replicate of the six samples were collected. For data entry and analysis Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) and its tools were used. The coefficient of variance of each sample, as well as the minimum-maximum range of the percentage of activated cells and their MFI was assessed for each of the used stimuli.

Results

Figure 1 shows the "live gate" (viability assessment) set on leukocyte DNA which is meant to distinguish between those events which have at least the same DNA content as a human diploid cell thus excluding aggregates of bacteria and fungi having the same scatter light properties as the leukocytes. Leukocyte viability decreases as burst oxidation intensity triggered by the used stimuli increases: viability of the negative control tube (97.87%) is followed by opsonized *E. coli* as particulate stimulus (70.43%) and then by the protein kinase C ligand PMA as high stimulus (45.93%).

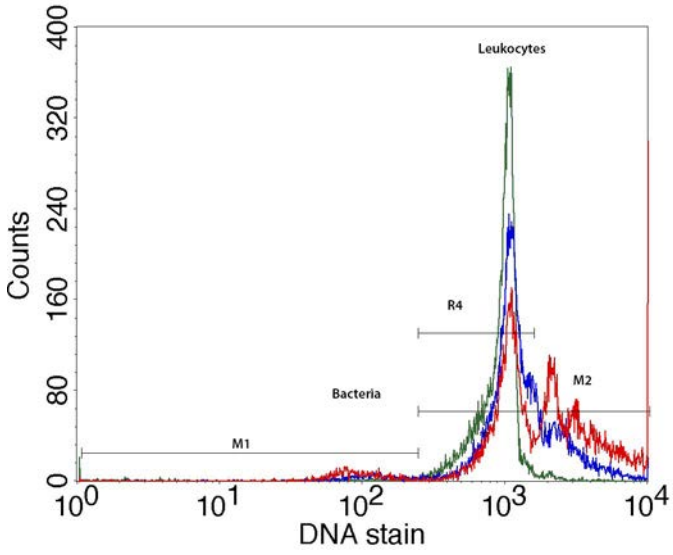

Fig. 1. Viability of assessed leukocyte population of the control sample (green), compared to the cell viability of the samples that were exposed to *E. coli* (blue) and PMA (red). A "live" gate (M2) was set on those events that have at least the same DNA content as a human diploid cell with the purpose of precluding from analysis bacteria or fungi (M1) which had the same scatter light properties as the leukocytes.

Figure 2 row 1 presents typical FSC/SSC dot plot sets of one of the tested subjects. The gate is set on granulocytes and monocytes stimulated with *E. coli* (1A), fMLP (1B), PMA (1C) and *C. albicans* (1D).

Figure 2 row 2 displays the degree of granulocyte (R2) and monocyte (R3) activation (recruitment) and production of ROS when using the same stimuli as mentioned above. *E. coli* (2A) and PMA (2C) trigger a similar intense PBP activation, while the burst activation triggered by the unopsonized *C. albicans* (2D) is similar to the one generated by the presence of low fMLP stimulus (2B). The burst activity (amount of DHR 123 cleaved substrate) of the two studied leukocyte populations was also quantified as mean fluorescence intensity (MFI) for each stimulus as depicted in *Figure 2* 3A, 3B, 3C, 3D for granulocytes, and *Figure 2* 4A, 4B, 4C, 4D for monocytes.

Table I shows the activation range of granulocytes and monocytes as percentages and MFI when verifying in our

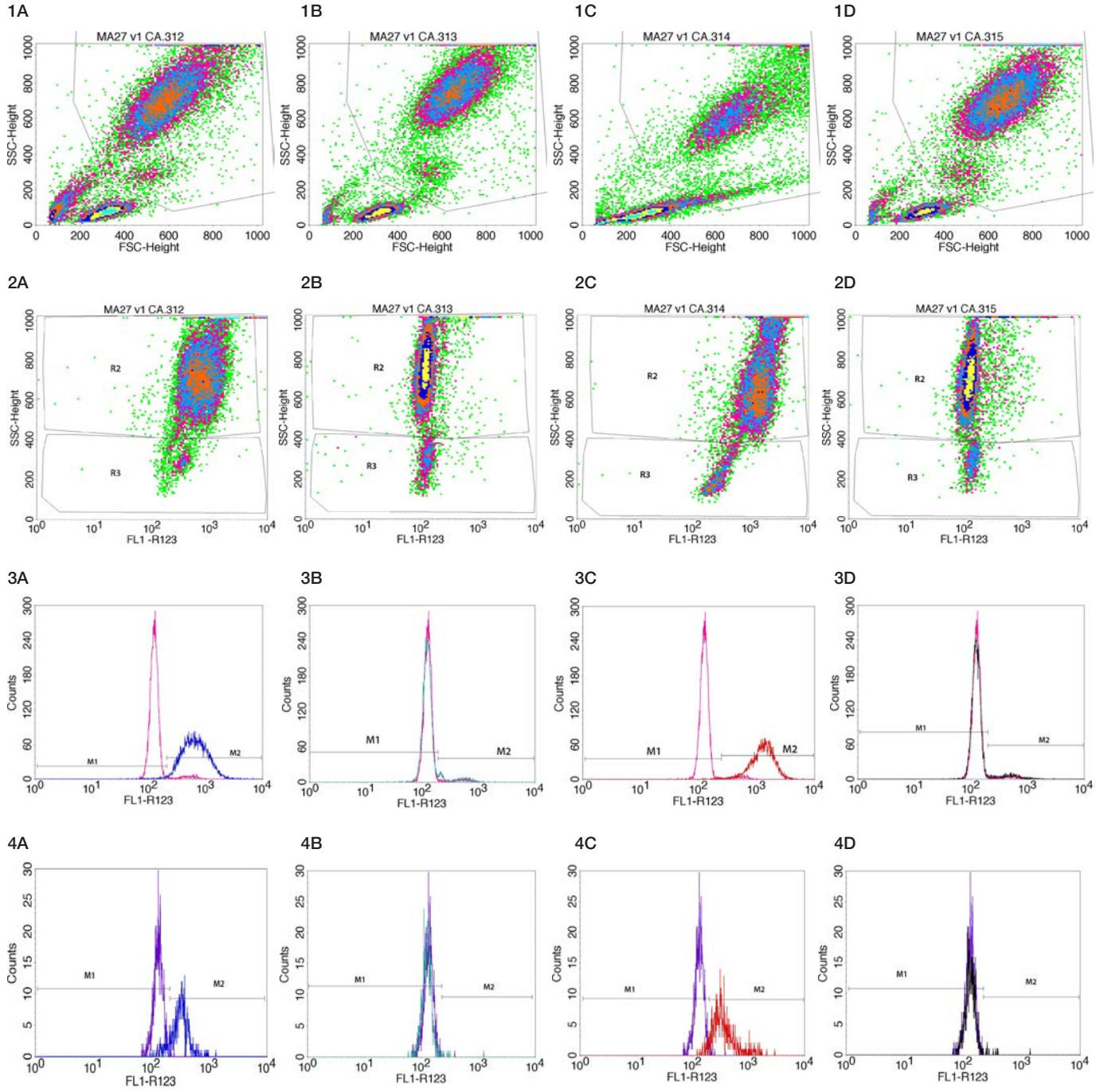

Fig. 2. Flow cytometry analysis results. Row 1 displays the typical FSC/SSC dot plots when adult PBP cells are stimulated with *E. coli* (1A), fMLP (1B), PMA (1C) and *C. albicans* (1D) (gate set on granulocyte and monocyte populations). Row 2 displays the dot plot lin SSC/log FL1-R123 of test samples stimulated with *E. coli* (2A), fMLP (2B), PMA (2C) and *C. albicans* (2D). Row 3 shows the typical FL1-DHR123 histogram that depicts granulocyte activation of the control sample (pink) compared to activation triggered by *E. coli* (blue) (3A), fMLP (green)(3B), PMA (red)(3C) and *C. albicans* (black) (3D). Row 4 illustrates the typical FL1-DHR123 histogram that shows monocyte activation of the control sample (violet) compared to burst activation triggered by *E. coli* (blue) (4A), fMLP (green)(4B), PMA (red)(4C) and *C. albicans* (black) (4D).

laboratory the intra-assay precision, as well as the average CV that resulted after testing in triplicate each of the 6 whole blood samples as recommended by the producers.

The activation range of granulocytes and monocytes, though having a broader lower limit in our laboratory conditions, was similar to the one described by the producer in the presence of *E. coli* (granulocytes: 78.45-99.43% versus 99.6-99.95%, average %CV of 1.53% versus 0.1%, monocytes: 54.63-92.33% versus 81.80-96.67, average %CV 6.92% versus 1.1%).

We obtained similar ranges of activation as the producers did for fMLP and PMA in the case of granulocytes, while for monocytes data for these stimuli were not provided by the producer.

When assessing the PBP burst oxidation triggered by *C. albicans*, our data revealed acceptable variation of mean %CV for granulocytes (6.46% for oxidizing cells and 7.54% for MFI). Due to low monocytes activation, during the 20 minutes incubation time, the resulting average %CV showed higher but acceptable values for validation (8.80 % for oxidizing cells and 11.61% for MFI).

Discussion

The *Phagoburst ™ (Glycotope Biotechnology)* kit that we used for our study is already validated for in vitro diagnostic testing with the above mentioned stimuli. According to professional standards, it is recommended that the performance criteria of each test to be verified in the user's own laboratory conditions. We performed this verification and then we tried to validate a new fungal stimulus by quantifying the intra-assay imprecision and the ranges of neutrophil and monocyte burst activation triggered by various stimuli.

According to the Practice guidelines from the ICSH and ICCS-part V- Assay performance criteria [11] recommended for validation of cell-based fluorescence assays, for the quantification of the intra assay imprecision, we used the same assay matrix (whole blood) originating from 6 patients (a minimum of 5 samples being required) tested in triplicate in a single analytical run [12]. We used samples only from healthy patients due to the laboratory's inability to access samples from patients with altered oxidative burst activity such as chronic granulomatous disease. We made this choice in order to be also able to compare our laboratory's results to the ones provided by the producer of the commercial kit which tested only healthy subjects.

The mean and %CV for each sample tested in triplicate was calculated. As recommended [12], percent CV (%CV), rather than standard deviation (SD) was used as acceptance criteria due to the fact that %CV normalizes variations at lower levels of event detection as it happened in our case of monocyte activation when using the low stimuli fMLP and *C. albicans*.

The level of acceptable imprecision in flow cytometry techniques for an reportable result depends upon the frequency of the population and the total number of events acquired [13]. As stated by the guidelines [11], a desirable target for assay imprecision is a CV of less than 10%, while for less abundant populations (frequency at 1:1000 (0.1%)) a CV of less than 20% is acceptable [14].

Table I. Verification performance parameters of the Phagoburst™ Glycotope Biotechnology commercial kit

Cell Type	Stimulus		Oxidizing cells (%)		GeoMean FL1 (MFI)	
			Producer	Own Laboratory	Producer	Own Laboratory
Granulocytes	*E. coli*	(min-max)	99.6-99.95	78.45-99.43	154.5-395.75	400-735
		mean		91.96		538
		average CV	0.1	1.53	4.8%	6.84%
	fMLP	(min-max)	1-10	1.53-12.95		320-496
		Mean		6.35	NA	387
		average CV	NA	18.61		4.08%
	PMA	(min-max)	98-100	95.83-99.77	300-1000	907-1528
		Mean		98.57		1215
		average CV	NA	0.78	NA	6.21%
	C. albicans	(min-max)		2.18-8.80		371-519
		mean	NA	5.68	NA	458
		average CV		6.46		7.54
Monocytes	*E. coli*	(min-max)	81.80-96.67	54.63-92.33	49.60-88.65	278-349
		mean		69.18		307
		average CV	1.1	6.92	6.5%	5.57%
	fMLP	(min-max)		0.61-4.52		236-410
		mean	NA	2.14	NA	283
		average CV		56.59		18.23
	PMA	(min-max)		29.15-95.89		285-383
		mean	NA	70.89	NA	356
		average CV		7.57		4.41
	C. albicans	(min-max)		1.04-10.63		239-347
		mean	NA	3.6	NA	299
		average CV		8.80		11.61

CV= coefficient of variance (mean of the %CV), min=minimum value, max= maximum value, NA= non-available, MFI=mean fluorescence intensity

The results obtained in our own laboratory conditions for the intra-assay precision are acceptable according to the guidelines [11] when assessing the %CV of oxidizing granulocytes and monocytes in the presence of *E.coli*, PMA and C. *albicans*. Also the ranges of values for oxidizing leukocytes that we obtained when assessing imprecision were similar to the ones described by the producer though having an acceptable lower reference range.

We did get a high %CV for granulocyte and monocyte activation as well as a high %CV for monocyte MFI when fMLP was used as stimulus. This situation that can be easily explained by the fact that considering the very low degree of PBP activation, very small variations generated large %CVs. Nevertheless, a value for fMLP %CV hasn't been provided for comparison not even by the kit manufacturer. A borderline value of 11.61%, slightly above the acceptable criteria, was also obtained for the MFI in the case of *C. albicans*.

When examining the MFI, in our laboratory conditions and flow cytometer settings, the value ranges, thought proportional to the ones described by the producer, were higher. It is one of the producer's recommendations that laboratories should establish their own normal reference ranges using their own testing conditions.

In the experiment we conducted in healthy adults, the particulate opsonized stimulus *E. coli* generated a similar activation of the PBP as the high stimulus (PMA), though with larger lower ranges in our laboratory conditions. MFI in our experiment, as described by the producer, reached higher values when PMA was used in comparison to the opsonized particulate stimulus *E. coli*.

The burst activation generated by incubation with *C. albicans* was similar in granulocytes and slightly lower in monocytes than the one generated by the low stimulus (fMLP).

C. albicans can reside as a lifelong commensal on or within the human host for a long time. Still, literature describes a number of pathogenic mechanisms that render *C. albicans* virulent when alterations in the host environment occur [15]. One of these mechanisms is represented by the robust stress response *C. albicans* displays against the oxidative and nitrosative stresses of the phagocyte cells [16,17]. Studies show that *C. albicans* mutants that lack genes that encode regulators of stress response or detoxifying enzymes have an attenuated virulence [18]. The ability of *C. albicans* to produce antioxidant enzymes like catalase Cta1 as well as the intracellular and extracellular superoxide dismutases (Sods) to counteract the respiratory burst [17,19], represents a viable explanation of the low activation and ROS production by neutrophils and monocytes in our study. Previous studies have shown that Sod1 interacts with macrophages while Sod2 is required to resist neutrophil attack [19]. Sod4, Sod5 [20] and Sod6, along with the catalase Cta1 detoxify extracellular ROS produced by macrophages [21].

According to previous studies described by Mayer at al. [17], due to quorum sensing, a low yeast density (<10^7 cells/mL), as in our case (1-5 x 10^6 cells/mL), favours hyphal formation. Though we did not submit the *C. albicans* yeast cells to any inactivation process prior to whole blood exposure, the smears performed after 10 minutes of incubation at 37°C showed no budding, pseudohyphae or hyphal growth. Still, hyphae could have formed during the next 10 minutes of incubation with the DHR123 prior to burst oxidation assessment. Studies have shown that regardless of the fungal morphology, Sods are expressed: yeast cells express Sod4 while hyphal forms express Sod5. Neutrophils also induce the expression of Sod5 although they inhibit the yeast-to-hyphal formation in *C. albicans* [19,20].

Other studies have shown the enhanced capacity of the human neutrophils from healthy patients in the presence of opsonins to inhibit germination of *C. albicans* into clusters of hyphae in overnight assays as well as to kill *Candida* conidia (2 hours). The killing of the unopsonized *C. albicans* was found to be dependent solely on the complement receptor 3 (CR3) and the signaling proteins phosphatidylinositol-3-kinase and caspase recruitment domain-containing protein 9 (CARD9), but completely independent of NADPH oxidase activity, as opposed to opsonized *C. albicans* whose killing was dependent upon production of ROS by the NADPH oxidase system [22].

Consequently, all these mechanism could explain the low production of ROS by phagocytes in the presence of unopsonized *C. albicans.*

Study limitations

The lack of supplementary opsonins in our study in the case of *C. albicans* stimulus, coupled with the relatively short incubation time (20 minutes), compared to other studies that used isolated PBMC might have led to a lower degree of burst activation than in the case of opsonized *E. coli*.

Conclusions

The performance parameters for the *Phagoburst ™ (Glycotope Biotechnology)* kit obtained in our laboratory compared to the ones provided by the producer as well as the professional guidelines allow us to safely use the kit for burst oxidation assessment in human PBP. Our study suggests also that introducing an extra fungal stimulus for burst oxidation assessment could provide additional information regarding the non-specific cellular immune response, particularly in patients at risk for fungal bloodstream infection.

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Conflict of interests

The authors declared no potential conflicts of interest with respect to this research, authorship and/or publication of this article.

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