Novel flow cytometry method for the detection of neutrophil activation and degranulation in peripheral human whole blood

Fidelta
A Galápagos Company

Denis Polančec*, Mila Vrančić*, Sonia Dupont, Katarina Orešković***, Vesna Eraković Haber***

* Fidelta d.o.o. Prilaz baruna Filipovića 29, Zagreb, HR-10000, Croatia;
** Galapagos SASU, 102 Avenue Gaston Roussel, 93230 Romainville, France;
*** Department of Biotechnology, University of Rijeka, Radmile Matejčić 2, 51000 Rijeka, Croatia.

E-mail: denis.polancec@glpg.com

Introduction

The aim of the study was to develop a simple, robust and reliable flow cytometric method for the detection of activation and degranulation of neutrophil granulocytes in human whole blood.

Excessive migration of neutrophilic granulocytes into diseased tissue is a known hallmark of a number of chronic inflammatory diseases. Neutrophil migration is dependent on expression of several adhesion molecules, which enable neutrophil interaction with endothelial and microbial ligands. Among these, CD66b is a granulocytes-specific activation antigen involved in granulocyte adhesion and transmembrane signalling. In addition to its surface expression, CD66b is contained in neutrophil specific granules and can be upregulated by inflammatory stimuli due to degranulation. The same is true for CD11b, which is contained in specific granules, gelatinase granules and secretory vesicles. Since increased CD11b expression does not necessarily correlate to augmented CD11b-adhesive capacity we have used an antibody specific for conformational activation of CD11b, which reveals a neo-epitope within the N-terminal ligand-binding I domain known as CBRM1/5 or activation-specific epitope (AE). An increased expression of conformationally active CD11b is seen on circulating neutrophils during systemic acute inflammatory states and given its functional significance *in vitro*, it is considered to be more relevant for the pathophysiology of neutrophil-mediated tissue injury than total CD11b levels.

Materials and Methods

Whole blood samples taken from healthy volunteers were stimulated with various pro-inflammatory agents: TNFα, bacterial lipopolysaccharide (LPS), N-formyl-methionyl-leucyl-phenylalanine (fMLP) and IL-8. Four different concentrations for each of the stimuli were used. Pro-inflammatory agents were dissolved in RPMI1640 medium at 2x concentration, added to whole blood at a 1:1 ratio, gently mixed and the samples were put in a CO_2 incubator at 37 °C for 120 minutes.

Immunophenotypization of whole blood samples was performed using fluorochrome-labeled antibodies from eBioscience (CD11b [AE] and CD16) and BD Bioscience (CD45 and CD66b). Unspecific binding of fluorochrome-labeled antibodies was blocked by normal Mouse IgG (Invitrogen). Upon addition of blocking IgG, tubes were mixed briefly and gently, and incubated at 4 °C for 10 min, protected from light. Subsequently fluorochrome-labeled antibodies were added, samples were gently mixed and incubated at 4 °C for 30 min, protected from light. Next, 1x FACS™ Lysing solution (BD Bioscience) was added according to manufacturer instructions, and samples were vortexed briefly and incubated at RT for 10 min, protected from light. Cells were pelleted by centrifugation at 550 g/4 °C/10 min, supernatants were discarded, cell pellets were resuspended in remaining buffer and 2 mL of Wash/Staining buffer (W/S buffer; PBS + 2% FBS) was added. Cells were then pelleted by centrifugation at 550 g/4 °C/5 min, supernatants was removed, cell pellets were resuspended in 300 µL of W/S buffer and analysed on FACScan/CytekDev DxP8 flow cytometer using FlowJo Collector's edition software. The expression of CD45 and CD16 was used to gate neutrophils, and mean fluorescent intensities (MFI) for all four receptors were determined on $CD45^{+}CD16^{high+}$neutrophils.

Results I

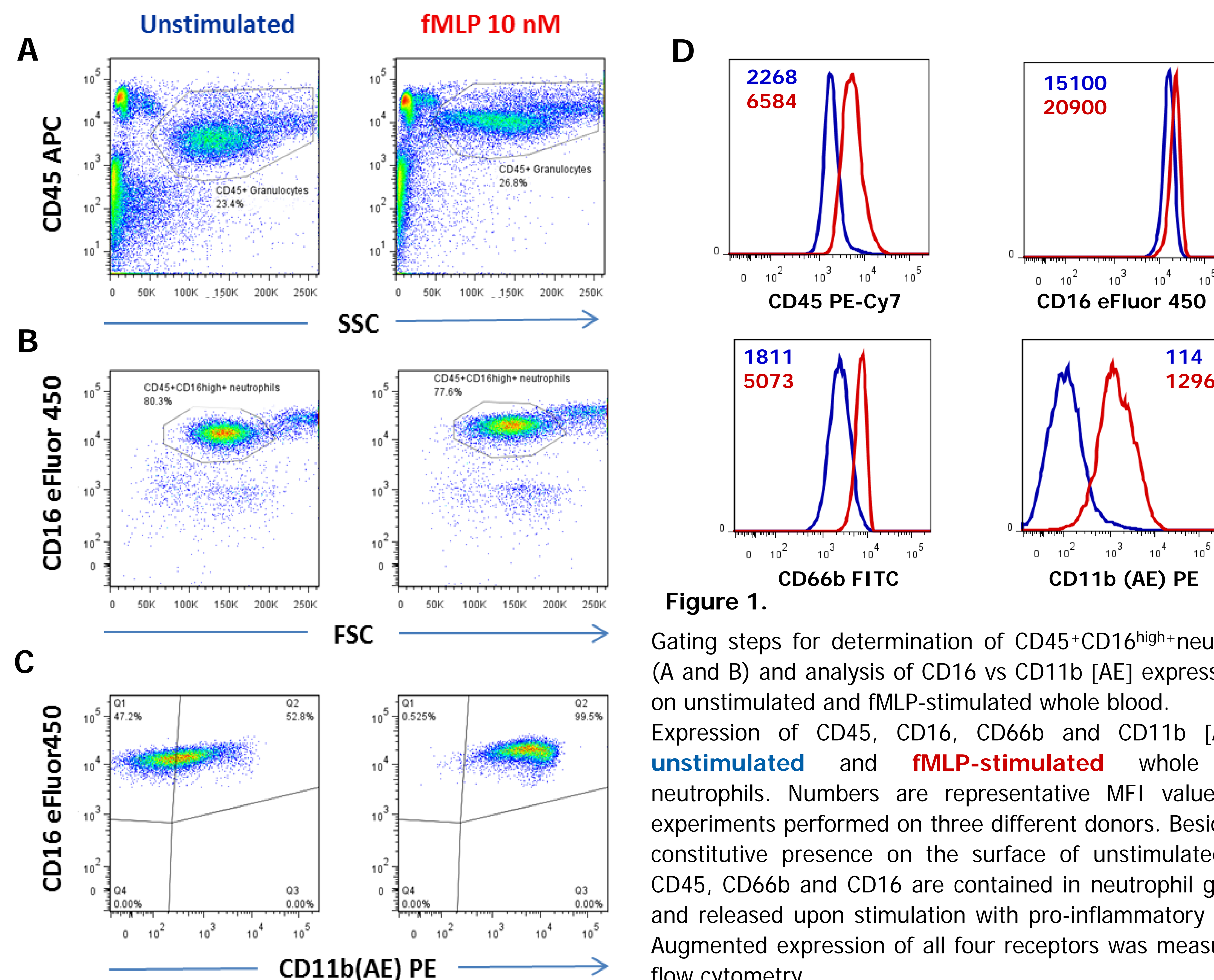

Figure 1.

Gating steps for determination of $CD45^{+}CD16^{high+}$neutrophils (A and B) and analysis of CD16 vs CD11b [AE] expression (C) on unstimulated and fMLP-stimulated whole blood.

Expression of CD45, CD16, CD66b and CD11b [AE] on **unstimulated** and **fMLP-stimulated** whole blood neutrophils. Numbers are representative MFI values from experiments performed on three different donors. Beside their constitutive presence on the surface of unstimulated cells, CD45, CD66b and CD16 are contained in neutrophil granules and released upon stimulation with pro-inflammatory agents. Augmented expression of all four receptors was measured by flow cytometry.

Results II

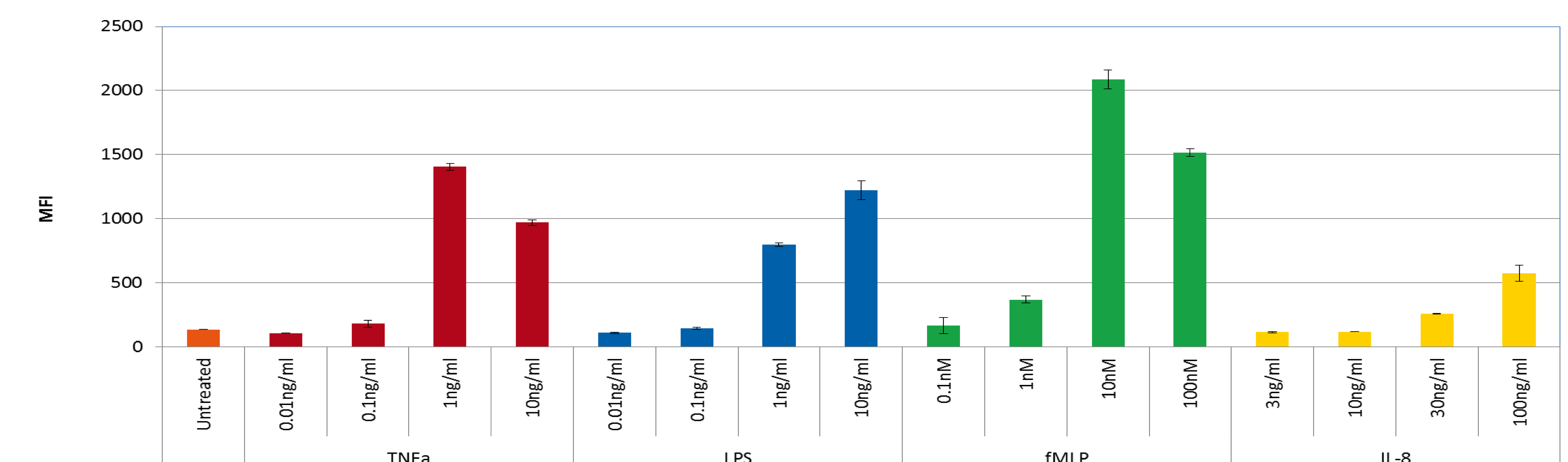

Figure 2.

Dose-dependent effect of pro-inflammatory stimuli on CD11b [AE] expression on peripheral whole blood neutrophils. Representative data from one of the experiments performed with blood taken from three healthy volunteers are shown. Error bars represent SDEV from biological triplicates.

Conclusions

The flow cytometric measurement of increased expression of CD45, CD16, CD66b and CD11b [AE] receptors on human neutrophils in stimulated whole blood is a good and valuable tool for detection of neutrophil activation and degranulation.

Changes in expression of CD11b [AE] upon various pro-inflammatory stimulations were most robust with the highest signal-to-noise ratio.

A reliable and reproducible *in vitro* whole blood assay was developed for pharmacodynamic studies of potential new drugs which exert anti-inflammatory effect by inhibition of neutrophil activation.

References:
1. Witko-Sarsat et al. 2000. Lab Invest. 80:617-653.
2. Mazzone et al. 1995. Haematologica. 80:161-17
3. Borregaard et al. 2007. Trends Immunol. 28:340-345.
4. Orr et al. 2007. J Leukocyte Biol. 82:1115-1125.

Poster available online at:
www.fidelta.eu

© Copyright 2013 Galapagos NV