Flow cytometric measurement of the CD11b Activated Epitope [AE] expression on human neutrophils in whole blood as a tool in drug development process

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Introduction

The aim of the study was to investigate the influence of GLPG0974, a novel, selective antagonist of human free fatty acid receptor 2 (FFA2), on short chain fatty acid (SCFA)-induced activation of neutrophil granulocytes in human whole blood. SCFAs, like acetate and propionate, induce neutrophil migration by activating FFA2 on neutrophils. A growing body of evidence suggests that FFA2 blockade may be beneficial in the treatment of neutrophil-dominated inflammation. In order to support the clinical development of GLPG0974, the task was to establish a robust assay for the measurement of FFA2 inhibition in whole blood. One of the approaches was immunophenotypization of SCFA-stimulated whole blood samples by flow cytometry using surface expression of CD11b[AE] and CD66b on neutrophils as markers of neutrophil activation and degranulation, respectively. Preliminary experiments (data not shown) revealed that SCFAs cannot induce neutrophil activation in whole blood *in vitro* unless the blood leukocytes are primed with another stimulus. Therefore, acetate- and propionate-induced CD11b[AE] overexpression was measured in LPS-, IL-8, fMLP- or TNFα-primed blood. All priming agents were used with or without Cytochalasin B (CyB). The most robust response was observed when acetate was used in combination with TNFα/CyB-primed blood, while propionate exibited lower potency (data not shown). Stimulation was more pronounced for CD11b[AE] than CD66b.

Materials and Methods

Human whole blood was obtained from healthy volunteers, with CPDA1 (citrate phosphate dextrose adenine) used as an anticoagulant. Stock solutions of the compound were prepared in DMSO, while working dilutions and DMSO controls were prepared in RPMI1640 cell culture medium. 100 µL of RPMI1640, with or without TNFα/CyB (2x final concentrations: TNFα 4 ng/ml, CyB 40 µg/ml), and GLPG0974 or DMSO (at 2x final concentrations) was added to sterile 5 mL polypropylene tubes, followed by 100 µL aliquots of undiluted blood. The samples were gently mixed and incubated for 15 min in a CO_2 incubator at 37 °C. After 15 min, SCFAs were added at 10 mM final concentration, samples were gently mixed and incubated for 2 h in a CO_2 incubator at 37 °C. In another set of experiments, the compound was added 15 min before the priming agents in order to investigate its effects on the priming itself. After 15 min, LPS, TNFα, IL-8 or fMLP were added, samples were incubated for 15 min and subsequently stimulated for 2 h with SCFAs, or left unstimulated. 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 antibodies was blocked by normal mouse IgG (Invitrogen). Upon adding the 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 mixed briefly and gently, and incubated at 4 °C for 30 min, protected from light. Next, 2 ml/sample of 1 x FACS™ Lysing solution (BD Bioscience) was added, the samples were vortexed briefly and incubated at RT/10 min protected from light. Cells were pelleted by centrifugation at 550 g/4 °C/10 min, supernatants was discarded, cell pellets were resuspended in the 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 and 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. Mean fluorescent intensity (MFI) for CD11b[AE] receptor was determined on $CD45^{+}CD16^{high+}$neutrophils and percentage of inhibition was calculated.

Results

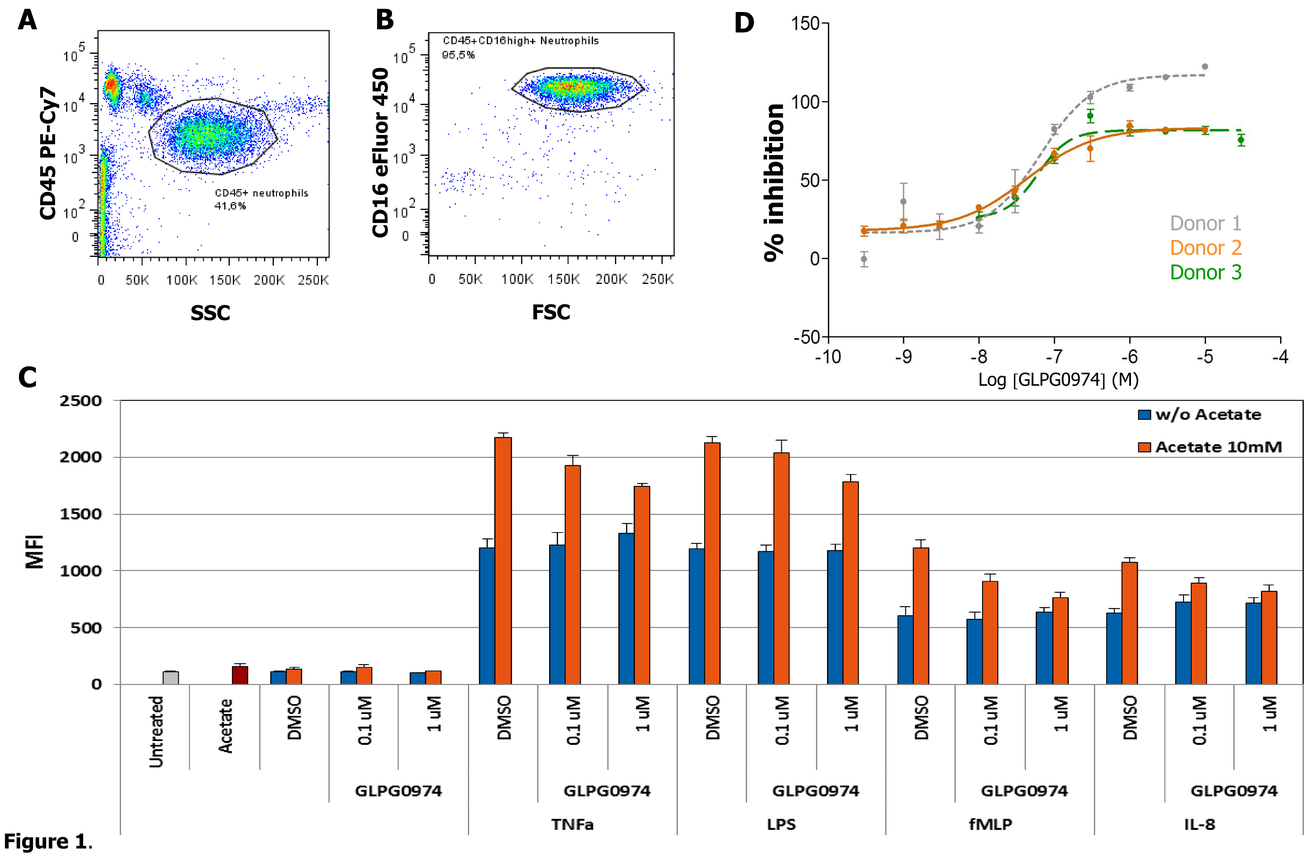

Figure 1.
Gating steps for determination of $CD45^{+}CD16^{high+}$neutrophils (A and B). GLPG0974 inhibited acetate-induced augmentation of neutrophil expression of the CD11b[AE] in TNFα-, LPS-, fMLP- and IL-8-primed human peripheral blood, without affecting the responses induced by TNFα-, LPS-, fMLP and IL-8 priming. GLPG0974 dose-dependently inhibited acetate-induced overexpression of CD11b[AE] on TNFα/CyB-primed human peripheral blood neutrophils (D). Representative data from experiments performed with blood taken from three healthy volunteers are shown (C and D). Error bars represent SDEV from biological triplicates. CD66b was inhibited as well, but less potently than CD11b[AE] (data not shown).

Conclusions

GLPG0974 is a specific inhibitor of acetate-induced activation and degranulation of neutrophils in human peripheral blood initially primed/conditioned by various pro-inflammatory stimuli.

A reliable and reproducible whole blood assay was developed for pharmacodynamic assessment of FFA2 antagonists in clinical trials.

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