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Pitfalls in flow cytometric analyses of surfactant-exposed human leukocytes

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ABSTRACT

Background: Surfactant replacement treatment is the standard of care for the prevention and treatment of neonatal respiratory distress syndrome in preterm infants and may also improve oxygenation in acute respiratory distress syndrome in children, adolescents and adults. Beside surface tension- and mechanical shear-reducing functions, natural surfactants have been ascribed immunomodulatory capacities. Current *in vitro* studies on immunomodulatory effects of pulmonary surfactant preparations on human leukocytes rely on ELISA, Western blot and polymerase chain reaction. Data obtained by flow cytometry are missing, so far, most likely due to confounding phospholipid residues. Intracellular cytokine flow cytometry in surfactant-exposed immune cells would provide information on pro- and anti-inflammatory immunomodulation at the single-cell level and would allow for integrating detailed immunophenotyping, functional assays and assessment of viability.

Aim: We implemented a flow cytometry protocol for reliable quantitative assessment of *in vitro* intracellular cytokine production in surfactant-exposed human lymphocytes (CD4$^+$) and monocytes (CD14$^+$).

Methods: Two different permeabilization techniques were tested for their ability to provide intracellular cytokine staining in surfactant-exposed CD14$^+$ monocytes and CD4$^+$ lymphocytes. Both a commercially available solution containing saponin and ice-cold methanol were used as permeabilization reagents.

Results: For both cell types, flow cytometry following saponin-based permeabilization revealed pronounced unspecific fluorescence signals in surfactant-exposed samples overlapping with the fluorescence spectra of the majority of conjugates. Autofluorescence of surfactant phospholipid particles interfered significantly with reliable quantification of fluorochrome-specific signals and conclusive analysis. Implementation of a methanol-based permeabilization protocol resulted in the elimination of confounding non-cell particle signals allowing for an accurate quantification of intracellular cytokine production.

Conclusion: Reliable detection of intracellular cytokines by flow cytometry may be challenging in surfactant-exposed cell samples due to significant autofluorescence of aggregated phospholipid particles. This issue has been addressed for the first time and may be of high relevance for all types of surfactant research. We demonstrate that a methanol-based permeabilization approach completely removes interfering fluorescent surfactant micelles and allows for correct evaluation of data. The successful removal of confounding surfactant phospholipids opens up a wide variety of multiparameter flow cytometry; a method that has not been applied in the field of surfactant research, yet.

1. Introduction

Flow cytometry offers high-speed quantitative multiparameter analysis of intra- and extracellular epitopes and simultaneous evaluation of cell viability. It is a powerful tool for quantitative and qualitative measurement of key proteins involved in immunological processes. Current polychromatic flow cytometry (PFC) approaches often use 8 till 11 dyes excited by three different laser lines [1,2]. Moreover, improvements in both fluorochromes and

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instrument hardware already provide high-dimensional PFC analysis using 15 till 18 dyes excited by 5 different lasers [3,4]. To our knowledge, the potential of this technology has been hardly exploited for surfactant preparations and surfactant phospholipids, yet. Data on immunomodulatory capacities of pulmonary surfactant mainly rely on quantitative and semi-quantitative analysis, such as enzyme-linked immunosorbent assay (ELISA) and Western blot [5–11], both allowing only for analysis of proteins from cell culture supernatants. Pulmonary surfactant replacement therapy has become essential in neonatal respiratory distress syndrome (RDS) [12] and is often used as an additional therapy in acute respiratory distress syndrome in adolescents and adults. Surfactant directly interacts with the alveolar-capillary unit and strongly influences its biophysical characteristics. A beneficial regulation of pro- and anti-inflammatory cytokine release within the alveolar space would represent a desirable effect of surfactant preparations. There are numerous studies analyzing surfactant activity and composition in this context [5,9,11,13–18]. However, none of the *in vitro* studies provides data obtained by flow cytometry analysis. We hypothesize that a relevant number of study approaches failed to obtain reliable data in the presence of confounding phospholipids. In fact, pulmonary surfactant being a complex lipoprotein mixture, consists of ~90% lipids, mainly phospholipids (PLs) comprising phosphatidylcholines (PCs) and phosphatidylglycerols (PGs), and of ~10% proteins, mainly surfactant proteins (SP) A, B, C and D [11,14,16,19–21].

In the present study, we aimed at implementing and validating cytokine flow cytometry in $CD14^+$ monocytes and $CD4^+$ lymphocytes exposed to different surfactant preparations to investigate intracellular cytokine response.

2. Material and methods

2.1. Surfactant preparations

Curosurf® is a natural surfactant derived from porcine lungs. It was obtained from Chiesi Farmaceutici S.p.A. (Parma, Italy). In addition to Curosurf®, we tested the following surfactant phospholipid preparations, that had been a kind gift of Prof. Tore Curstedt, Sweden: A mixture of dipalmitoyl-phosphatidylcholine (DPPC; 67.3%), palmitoyl-oleoyl-phosphatidylglycerol (POPG; 28.8%), and SP-B and SP-C analogs (3.8%) (DPPC + POPG + SP-B/C), as well as a mixture of DPPC (48.1%), POPG (9.6%), SP-B and SP-C analogs (3.8%), and egg yolk L-α-phosphatidylcholine (L-α-PC; 38.5%) (DPPC + POPG + SP-B/C + eggyolkPC). In preliminary dose–response experiments (100 μg/mL–1 mg/mL), 100 μg/mL of Curosurf® and the given surfactant phospholipid preparations had maximally affected cytokine release without inducing apoptosis or cell death.

2.2. Antibodies

Antibodies to the surface epitopes CD14 (clone HCD14, Pacific Blue-conjugated), CD16 (clone 3G8, PE-conjugated) and CD4 (clone OKT4, APC-conjugated) were all purchased from BioLegend (San Diego, CA). For intracellular cytokine staining, antibodies to TNF-α (clone MAb11, PerCP-Cy5.5-conjugated), IL-1β (clone H1b-98, Alexa Fluor 647-conjugated), IL-8 (clone E8N1, Alexa Fluor 488-conjugated) and IL-10 (clone JES3-9D7, PE-Cy7- or Alexa Fluor 488-conjugated), IL-2 (clone MQ1-17H12, PE-Cy7-conjugated) and IL-4 (clone MP4-25D2, PE-conjugated) were obtained from BioLegend, the antibody to IFN-γ (clone 4S.B3, PerCP-Cy5-5-conjugated) was purchased from BD Biosciences (Franklin Lakes, NJ). Table 1 illustrates the selected fluorochromes including excitation wavelengths and emission range.

2.3. Enrichment of peripheral blood mononuclear cells

Leukocyte concentrates (buffy coats) were obtained from apheresis products from anonymized healthy adult donors (consent and collection guidelines were in accordance with the Declaration of Helsinki). Peripheral blood mononuclear cells (PBMCs) were accumulated from the heparinized blood on Ficoll-Paque (LINARIS Biologische Produkte GmbH, Dossenheim, Germany) for 25 min at 530 × *g*.

2.4. Enrichment of $CD14^+$ monocytes and $CD4^+$ lymphocytes

$CD14^+$ monocytes and $CD4^+$ lymphocytes were further enriched by magnetic-activated cell sorting (MACS) targeting the antigen CD14 and CD4, respectively. Positive isolation of $CD14^+$ monocytes was performed using CD14 MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), the corresponding MidiMACS™ separator and LS type columns (Miltenyi Biotec) according to the manufacturers' instructions. Positive isolation of $CD4^+$ lymphocytes was performed using the Dynabeads® CD4 Positive Isolation Kit (Life technologies, Carlsbad, CA) according to the manufacturers' instructions. The purity of the isolated cells was determined by flow cytometry to be ≥90% for $CD14^+$ monocytes ≥97% for $CD4^+$ lymphocytes.

2.5. Cell culture and cell activation

$CD14^+$ monocytes and $CD4^+$ lymphocytes were resuspended in RPMI1640 (Sigma–Aldrich) supplemented with 10% fetal bovine serum alone, or 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Life technologies), respectively. 1×10^6 $CD14^+$ monocytes were either left unstimulated or stimulated with 100 ng/mL LPS (*E. coli* 055:B5, Sigma–Aldrich) and 100 μg/mL Curosurf® or one of the given surfactant preparations in 24 well plates (Greiner, Frickenhausen, Germany) for 14 h at 37 °C in a humidified atmosphere with 5% CO_2. In order to promote the accumulation of *de novo* synthesized cytokines in the Golgi apparatus, 10 μg/mL Brefeldin A (Sigma–Aldrich) was added to the cells.

2×10^5 $CD4^+$ lymphocytes were treated with 100 μg/mL Curosurf®, DPPC + POPG + SP-B/C, DPPC + POPG + SP-B/C + eggyolkPC or L-α-PC alone and simultaneously stimulated for 40 h at 37 °C in a humidified atmosphere with 5% CO_2 by using 96 well plates (Greiner) which were previously coated for 72 h at

Table 1
Overview of the fluorochromes used (bold) and autofluorescence detected, applying an eight-color staining approach.

No. Dyes	Laser [nm]	Fluorochromes	Detection range [nm]	Autofluorescence detected
8	488	**PE-Cy7**	750–810	yes
	488	**PerCP-Cy5.5**, PerCP	670–734	yes
	488	**PE**	564–606	yes
	488	**Alexa Fluor 488**, FITC	515–545	yes
	633	**APC-H7**	750–810	no
	633	**APC**, **Alexa Fluor 647**	640–680	yes
	405	**BD Horizon V500**, BV 510	485–535	yes
	405	**Pacific Blue**, BV 421	425–475	yes