Neisseria meningitidis Serogroup B Lipopolysaccharides Induce a Lower Pro-Inflammatory Effect within the Proteoliposome Used in Cuban Anti-Meningococcal Vaccines

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ABSTRACT

Neisseria meningitidis outer membrane vesicles or proteoliposomes (PLs) has been used as vaccines and adjuvant. Despite the presence of potentially toxic amounts of lipopolysaccharide (LPS), they have been shown to be safe, well tolerated, and immunogenic. This suggests that LPS-PL may have reduced LPS toxicity. We show here that the ability of PL to induce pro-inflammatory cytokine production in human U937 histiocytic cell line is significantly lesser than that of an equivalent concentration of purified LPS, thus confirming that certain components or physical properties of PL reduce the pro-inflammatory activity of their endogenous LPS. To investigate the mechanisms responsible for this protective effect, PLs were fractionated and assayed the ability of the resulting fractions to induce inflammatory cytokine expression. Several individual PLs fractions were more potent inducers of pro-inflammatory cytokine production than the unfractionated PLs. The majority of the pro-inflammatory activities appeared to be mediated by the presence of LPS in the fractions, as shown by the ability of an anti-CD14 antibody to block it. However, in two PL fractions, the production of IL-8 and to a lesser extent IL-6 was not inhibited by anti-CD14 treatment, indicating that pro-inflammatory components other than LPS could also be present in PL. Eight proteins present in the fractions were identified by n-terminal sequencing. Our results suggest that two of them PorB and particularly the RmpM protein may also contribute to the pro-inflammatory activity of *N. meningitidis* PL. Our results could support the development of PLs as vaccine adjuvant.

Key words: proteoliposome, *Neisseria meningitidis*, LPS, proinflammatory cytokines, adjuvant.

Running title: Proteoliposome of *Neisseria meningitidis* reduce LPS toxicity

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1. INTRODUCTION

Adjuvants play a determinant role in vaccine formulations[23,18]. As part of their immunostimulant effect, adjuvanted vaccines can over-stimulate the inflammatory mechanisms, with high production of TNF and other mediators, such as tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1 β), IL-2 and IL-6. They can generate a variety of symptoms, according to the quantity and the time they last in the plasma, ranging from a transitory hyperthermia to a state of shock, similar to that observed in sepsis[4]. Thus, adjuvant are determinant factors in the efficacy-toxicity ratio of the contemporary vaccines[2].

Outer membrane vesicles (OMV) also named Proteoliposomes-based vaccines of several types, based on different strains and specific antigens, have offered effective protection against B serogroup through the induction of mucosal and systemic bactericidal antibody responses mainly to outer membrane porins, PorA and PorB. *N. meningitides* (PLs) are regarded a valuable platform for a new generation of antimeningococcal vaccines[5]. PLs are extracted by detergents or obtained as blebs in the supernatant of *Neisseria meningitidis* serogroup B cultures. PLs has several proteins, lipoproteins, and lipopolysaccharide (LPS) or lipooligosaccharide included in the lipids constituents that form nanoparticles[8,9]. In mice and humans, PLs preferentially induces a cellular (Th1) immune response characterized by delayed-type hypersensitivity response, bactericidal and opsonic antibody subclasses, and interferon-IFN-γ) and IL-2 production. Specific IgE, anaphylaxis responses, IL-5, and IL-4 production, characteristic of Th2 response, are not induced by this PLs[20,21].

Innate immune response plays an essential role in the activation and direction of adaptive immunity mediated by Th1, Th2, Th17 and Tregs CD4+ as well as T CD8+ subpopulations [19]. Most pathogens share microbe-associated molecular pattern (MAMPs) which are invariant molecular structures recognized by the innate immune system also known as pathogen-associated molecular patterns, (PAMPs). The antigen presenting cells for naïve T cells contain a set of non-clonal pattern

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recognition receptors (PRR), which specifically binds to MAMPs. These receptors include Toll-like receptors (TLR), collectins, CD14, and mannose binding protein [16,32]. Among the members of the TLR family described, TLR4 is known as the receptor for some LPS and TLR2 for peptidoglycan and bacterial lipoproteins, particularly PorB of *N. meningitides*[30,15].

The multiple intracellular signaling events initiated by LPS recognition lead to the synthesis and release of several pro-inflammatory cytokines[29]. The participation of LPS in the uncontrolled inflammatory response leading to septic shock promoted research into the use of non-toxic LPS derivatives. They include natural analogues, physical or chemical modifications, synthetic derivatives, or genetic manipulations of synthetic pathways, particularly from *N. meningitidis*[33,6]. In spite of its toxic effect, native LPS are included in some highly immunogenic PLs successfully used as vaccines[27,5]. We hypothesized that the inclusion of native LPS in the lipid bilayer of PL is crucial for the reduction of LPS toxic effect. Therefore, the objective of this study was to determine whether the LPS as included in detergent-extracted PLs has reduced inflammatory capabilities compared to isolated LPS. We also aimed to elucidate the importance of major proteins from PLs in the production of inflammatory cytokines.

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2. MATERIAL AND METHODS

2.1 Preparation of Proteoliposome. PL was prepared from the outer membrane of serogroup B *N. meningitidis* strain Cu 385-83 (B:4:P1.19.15;L3,7,9) by detergent extraction[8,9]. Briefly, PLs were obtained from live bacteria by gentle extraction with 10% deoxycholate (DOC) (Merck, Darmstadt, Germany). Bacterial debris was removed by sequential centrifugation steps at 20 000 x g for 30 min. Following ultracentrifugation at 125 000 x g for 2 h, the pelleted PLs was homogenised in phosphate-buffered saline (PBS), pH 7.2, and nucleic acids were removed by enzymatic treatment with deoxyribonuclease and ribonuclease (5 µg/mL) (Merck, Darmstadt, Germany). PL was purified by gel filtration chromatography on Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden). Then, PLs were conserved in a buffer containing DOC. PL is a membrane vesicle that contains major outer membrane proteins (P1 [class 1, PorA] and P3 [class 3, PorB]), a complex of proteins from 65 to 95 kDa, LPS, and phospholipids. LPS inserted in PLs were measured by determination of 2-keto-3-deoxyoctonate by the thiobarbituric acid method[17] and represented the 4±2% of PL proteins content.

2.2 Lipopolysaccharide extraction and whole-cell lysate (WCL) preparations. LPS were extracted using a modification of the hot phenol-water method[34]. Briefly, the strain from stocks stored in skimmed milk (Merck, Darmstadt, Germany) at -70ºC, were grown in Mueller-Hinton broth (Oxoid) supplemented with Vancomycin-Colimycin-Nystatin (VCN) inhibitor (BioMerieux SA, Marcy I'Etoile-France) at 37ºC. After 6 h of rotator incubation, the cells were harvested. Bacteria were digested with DNase II (200 µg/mL) (Sigma) for 2 h at room temperature followed by-treatment with proteinase K (1 mg/mL) (Sigma) for 2 h at 60ºC and boiled for 10 min. Then, LPS were extracted using the hot phenol-water method. WCL of the same strain was also produced and adjusted to an OD of 0.1 at 600 nm.

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2.3 SDS-PAGE analysis and sequence analysis of PLs. SDS-PAGE analysis of PLs was performed on 12% polyacrylamide gels[14]. PLs and purified LPS were used at 10 and 2 µg *per* track, respectively. A control of WCL was included at 20 µL *per* track. The gels were stained with Coomassie to visualise proteins or silver stained to visualise LPS bands[31]. Low and high molecular weight standards were included in each gel (Bio-Rad). Molecular weights of PLs components were calculated using the molecular analyst program (Bio-Rad). For n-terminal sequencing, PLs and fractions 11-13 and 18 were separated on 12% polyacrylamide gels and then proteins were transferred onto nitro-cellulose, stained with Coomassiee and cut to sequence. The sequences obtained by n-terminal analysis were then compared with protein sequences in a data bank (www.ncbi.nlm.nih.gov) to elucidate the identity of the protein.

2.4 Electro elution of proteins and lipopolysaccharide from Proteoliposome. PLs were first separated using 12% polyacrylamide SDS-PAGE gels[11], and electroeluted (LKB 2014 Extraphor, Bio-Rad) at 100-200 V for 30 min. The fractions collected (5 mL each one) were then concentrated 5 times by using Amicon and the protein concentration of each fraction was determined by micro BCA protein assay (Pierce). Aliquots of each fraction were stored at -20ºC with or without 10% foetal calf serum (FCS) until use. The PLs fractions were analyzed by electrophoresis under reducing conditions, on 12% SDS polyacrylamide gels followed by Comassie blue or silver staining.

2.5 Stimulation assay using U937 cell line. The human U937 histiocytic cell line [ECACC cat no. 85011440] was used[28]. Cells were grown as a suspension in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, and 10% heated inactivated FCS (all Sigma) at 37°C in a 5% CO_2 humid atmosphere and subcultured every two or three days. The U937 cells were diluted ($5x10^5$ cells/mL) in medium with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma) and incubated 72 h at 37°C distributed 1 ml/well in 24 wells tissue culture plates (Nunclon, Nunc, Roskilde, Denmark). Prior to the stimulation assay the culture medium

containing PMA was removed and replaced with fresh medium without PMA. Cells were stimulated with PL (500, 250, or 125 ng of proteins/mL) diluted in DOC 0.5% or with each fraction (100 µL/well). The fractions were used in the same proportion as they were extracted from the proteoliposome's elution (170 ng in F4, F5, and F7; 500 ng in F8, F17, F18, and F19; 750 ng in F9 and F14; 900 ng in F10; 1300 ng in F13; 2500 ng in F11 and F12; and in the rest of the fractions they were less than 50 ng) in triplicate wells. LPS (10 ng/mL) or WCL (1:20 000) from *N. meningitidis* serogroup B were used as positive controls. DOC was used as negative control. Cells in six wells were not stimulated as additional controls. In parallel, plates were treated with an anti-CD14 mouse monoclonal antibody (mAb, Monosan, Uden, Netherlands) for 30 min before the addition of stimulants. Samples of culture supernatants were obtained before stimulation and 24 h post stimulation. At these time points, the cell culture and media were removed from the corresponding wells and centrifuged at 13 000 rpm for 5 min and the supernatants were harvested and stored at -20°C until tested.

2.6 Cytokine detection by ELISA. Cytokines were measured by capture enzyme-linked immunosorbent assay (ELISA) with antibody pairs and cytokine standards purchased from BD PharMingen. IL-1β was measured by using the human IL-1β ELISA set, BD OptEIATM, follow manufacturer instructions (BD PharMingen). For TNFα, IL-6, or IL-8 determinations, 96-well ELISA plates (Maxisorp Nunc Immuno plate, Nunc, Roskilde, Denmark) were coated overnight at 4ºC with 100 µL per well of a capture mouse anti-human TNFα-mAb (clone MAb11), rat anti-human IL6-mAb (clone MQ2-13A5) or mouse anti-human IL-8-mAb (clone G265-5) diluted in 0.1 M of sodium phosphate buffer (pH 6.0) at 2 µg/mL. After blocking with PBS supplemented with 10% foetal bovine serum (FBS) at 37°C for 1 h, 100 µL of supernatants from U937 cell line stimulation assays were added in triplicate and the plates were incubated at 37°C for 2 h. Serial twofold dilutions of recombinant human TNFα, IL-6, or IL-8 ranging from 10 ng/mL to 156 pg/ml were included as standards. Biotinylated mouse anti-human TNFα-mAb (clone MAb1), rat anti-human IL-6-mAb

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(clone MQ2-39C3) or mouse anti-human IL-8-mAb (clone G265-8); 100 µL/well at 1 µg/mL in PBS-10% FBS was then added for 1 h at 37° C, after which 100 µL of peroxidase-labelled streptavidin per well at 2.5 µg/mL (Sigma) in PBS-10% FBS was added for 45 min at room temperature. Ortho-Phenylenediamine (Sigma) (1 mg/mL in 0.2 M Na2HPO4 - 0.1 M citrate buffer) was used to develop the plates in the presence of H_2O_2. The reaction was stopped with 15 µL of 3 M H2SO4 per well and the optical density was read at 490 nm.

2.7 Statistic analysis. Significant differences between the means of two groups and different groups were determined by a Student T test and Tukey multiple comparison test using the Graph Pad Prism 4 software (Calif.), respectively. A p-value of <0.05 was considered statistically significant.

3. RESULTS

3.1 PLs induce lower pro-inflammatory cytokines production than equivalent amounts of pure lipopolysaccharide. The human U937 histiocytic cell line was differentiated and stimulated with PL or LPS at indicated concentrations and pro-inflammatory cytokine production (TNFα, IL-1β, IL-6, and IL-8) was measured by ELISA. LPS (10 ng/mL) induced production of all the pro-inflammatory cytokines tested. This effect was significant inhibited ($p<0.05$), as expected, by pre-treatment of the cells with anti-CD14 blocking mAb. Detergent extracted PLs obtained from *N. meningitidis* serogroup B was used as stimulant at 500, 250, or 125 ng/mL, containing LPS at the concentrations of 20, 10, or 5 ng/mL, respectively. PLs induced production of TNFα, IL-1β, IL-6, and IL-8 in a dose dependent manner (data not shown), but at a much lower level than purified LPS in all PLs concentrations tested. PLs at a dose of 250 ng/mL (with 10 ng/mL of inserted LPS) induced levels of cytokines dramatically lowers (TNFα, IL-1β, and IL-8 at ($p<0.001$) and IL-6 ($p<0.01$) than that induced by 10 ng/mL of purified LPS used alone (Fig. 1). After 24 h, the production of TNFα induced by PLs was much lower (93.8% less) than that seen with purified LPS. Similarly, induction

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of IL-1β, IL-6, and IL-8 were reduced by 94.3, 88.6 and 74.8%, respectively. Overall, PLs induces lower pro-inflammatory cytokines production than equivalent amounts of pure LPS from the same bacteria.

3.2 Fractionation of PL by slot elution and identification of major proteins by end terminal sequencing. Twenty fractions (F1-F20) from low to high molecular weight components were obtained by SDS-PAGE followed by slot electroelution separated according with their gel migration characteristics. SDS-PAGE and silver staining of the fractions showed that LPS was present in F1 and F2, while the principal meningococcal outer membrane proteins in the various fractions could be only tentatively identified at this stage. A protein with the migration characteristics of Class 1 (PorA) was detected in fractions F10 to F14, peaking at F13. Class 3 (PorB) appeared to be in F9 to F13 but predominated in F11 and F12, while Class 4 (rmpM) was mostly in F11, Class 5 (Opc) was in F9 and F10, and FrpB (70 kDa) was mostly in F18 (Fig. 2).

Eight major meningococcal outer membrane proteins present in the PL and its main fractions were identified using end terminal sequencing. Two of these appeared to be in more than one fraction obtained by slot elution (Fig. 3). We found that PLs contained surface protein A (NspA), identified as a band of approximately 18-22 kDa in fractions F4 and F5; Class 3 (PorB, 37-42 kDa) in F7 and F12; Class 4 (RmpM, 33-34 kDa) in F8 and F11; Class 5 (Opa, 22-33 kDa) in F9 and F10; Class 1 (PorA, 44-47 kDa) in F13; FrpB (70 kDa) in F17 and F18; and HmbR 76 kDa in F19. We found that the protein representation and concentration between independent *N. meningitidis* PLs vaccine batches are very similar (data not shown). Overall, PLs was separated in 20 fractions and there main proteins were identified still some of them were present in several fractions.

3.3 Fractionation of PL unmasks latent pro-inflammatory activity. Assays of cytokine production by differentiated U937 cells that had been stimulated by individual PLs fractions showed that several fractions induced dramatically higher levels of cytokine production than whole PLs. In

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particular, F1-F4, F8, F9, F11, and F18 were high inducers giving four distinct peaks of TNFα, IL-1β, and IL-6 production, while other fractions showed lower levels of induction. Additional fractions appeared to induce IL-8, although the pattern of IL-8 production broadly followed that of the other cytokines tested (Fig. 4). Taking in consideration that each fraction contains different protein concentration and that we used the same volume of each fractions to stimulate the differentiated cell line, a parallel experiment was conducted to evaluate these possible influence in the cytokine production. The independence of the different concentrations used was demonstrated where similar cytokine production behavior were found (data not shown).

Pre-incubation of the cells with anti-CD14 mAb (represented only the 3 picks of cytokine production) considerably reduced induction of cytokine production by most fractions. Remarkably, the effect of F11 (fractions that contain Class 4 protein, see below), in terms of IL-8 and F8 and F13-F14 for IL-1β productions were not inhibited, but enhanced, strongly suggesting that the pro-inflammatory activity of these fractions could not be due to LPS contamination. The effect of some other fractions, like the induction of TNFα by F8 and F10 or IL-8 by F5, F7, and F8 was not inhibited. In the case of fractions F8 and F10 TNFα production was inhibited by 33 and 27%, respectively. IL-8 production stimulated by F5, F7, and F8 was inhibited by the presence of anti-CD14 mAb by 31, 18, and 29%, respectively. These data suggest that certain PL fractions may have some pro-inflammatory activity, independent of LPS. In addition, when using F1 and F2 as stimulants there were only partial inhibition of IL-8 (40 and 24%, respectively) and IL-6 production (57 and 41%, respectively) after anti-CD14 treatment (Fig. 5).

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4. DISCUSSION

4.1 The LPS of PLs has significantly reduced inflammatory effects

The present evaluated the individual participation of each PL component in the pro-inflammatory effect of PL and to understand why native LPS, is well tolerated and immunogenic in small animals and human when it is inserted in PLs. Vaccine toxicity is associated with local inflammation and the production of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, and IL-8[3]. We first evaluated the production of these cytokines by differentiated U937 histiocytic human cell line stimulated with either purified LPS or PLs, which contains inserted native LPS. The production of pro-inflammatory cytokines by PLs was dose-dependent. A dramatic reduction in the production of TNFα and IL-6 and a reduction in the production of IL-1β and IL-8 were observed in samples stimulated with PLs, in comparison with samples stimulated by matching amounts of LPS. These results suggest that LPS inserted in PL have a different behaviour than when it is free. A recent study using The Hen's Egg Test on Chorioallantoic Membrane (HET-CAM) for a comparative evaluation of the irritating effect of different adjuvants, showed that PL and cochleates derived from *N meningitidis B* induced a low irritant effects with minimal local toxicity[1].

4.2 Some PLs fractions were more pro-inflammatory than LPS or whole PLs

We obtained twenty partially purified fractions from PL by slot elution after SDS-PAGE fractionation and tested their ability to stimulate differentiated U937 cells. Four distinct groups of fractions were found to induce cytokine expression. The first group corresponded to F1-F4, the second to F8-F9, the third to F11 and the fourth to F18. Similar results were obtained with each of the four cytokines evaluated. Fractions F1, F2 and, in some gels, F3 (data not shown) contained material detected by sugar silver staining that migrated in the same position as control LPS, indicating as expected, that the first three factions contained most the LPS component of the PLs preparations.

This indicates that the ability of F1 and F2 and probably F3 to strongly induce cytokine production is due to its LPS content. Indeed, in agreement with this conclusion, we found that the ability of the fractions to induce cytokine expression was almost completely blocked by anti-CD14.

In addition to LPS, the PLs contains lipoproteins and peptidoglycan which are activators of TLR2 and could therefore also be involved in PL-induced cytokine production[12]. We used CD14 depletion to test whether or not activation of cytokine production by the fractions occurred thought CD14 and TLR4 receptors. Although, LPS could be only detected by sugar silver staining of the gels in the first 3 fractions and not in the others, in most cases their ability to induce cytokine expression was also blocked by anti-CD14. This suggests that the ability of these fractions to induce cytokine expression can be owing to the presence of contaminating LPS. The remarkable cytokine induction by F18 in relationship with its low concentration and its completely blockage with anti-CD14 suggest that the protein present in this fraction is sticking more LPS than other proteins. The presence of FrpB, 70 kDa in this fraction in the PL might have implication in it immunopotentiator ability.

Interestingly, however, the ability of fractions F8 and F11 to induce IL-8 expression was not inhibited after CD14 depletion. This suggests that the two fractions may contain molecular species capable of inducing–pro-inflammatory response in a LPS independent fashion. This could also suggests that the activation of U937 histiocytic cell line to produce IL-6, TNFα, and IL-1β by PL can occurs by mechanisms other than for Il-8 production. This could imply the activation through different TLRs. The observation that high concentration of *Neisseria* antigen or *Neisseria* porins led to the production of pro-inflammatory cytokines independently of CD14 and TLR4 is in agreement with this interpretation. In addition, it is clear that Th1 adaptive immune responses require TLR signals[13]. The induction by PLs of a preferential Th1 response, previously reported by our group[5], might result from the presentation by the PL-LPS and lipoprotein in a physical form that limits their inflammatory properties while allowing Th1 induction. Thus is also suggested, based in our earlier findings, that LPS is essential to polarize the response towards a Th1 pattern both *in vitro* and in LPS

non-responder mice[26]. Quarkyi *et al.* demonstrated that LPS inserted in this kind of PL structures is less reactive by chromogenic LAL assay, less inductor of shock in animals and less pyrogenic in rabbits than purified LPS[25].

4.3 Identification of Class 4 protein as a major component in F11

In order to identify the proteins involved in the CD14-independent stimulation, ten protein bands from various fractions were sequenced and found to correspond to eight different proteins. In particular, the class 3 porin, PorB, which is a known TLR2 agonist[24,10], corresponds to a band whose distribution peaks in F12. However, the distribution of PorB does not match that of the unknown CD14-independent pro-inflammatory activity. Thus, PorB does not seem to account for the majority of the pro-inflammatory activity of fractions F7-F8 and F11-F12, at least in this system and concerning the cytokines used as inflammatory activity indicators. Our evidence suggests instead that certain unidentified components other than PorB or contaminating LPS may contribute to the ability of F7, F8, F11, and F12 to induce cytokine expression in a CD14-independent manner. Interestingly, the protein, RmpM, also known as the single component of class 4 protein[7], was distributed in two peaks corresponding to fractions, F7-F8 and F11-F12. Thus, the presence of RmpM in fractions correlated with their ability to induce the pro-inflammatory response in a LPS-independent fashion. This suggests that *N. meningitidis* RmpM protein might be able to activate a TLR. Further experiments using highly purified RmpM protein and cells derived from TLR-knock-out mice will be needed to confirm these data. Such experiments are, however, beyond the focus of the present article. It is worth noting here, however, that RmpM is less represented in our PLs than in others, which might contribute to explain some differences in the pro-inflammatory and protective behaviour of these preparations. Another characteristic that distinguishes the PLs of the Cuban vaccine studied here from others is the presence of FrpB, 70 kDa. A future comparison of these different PLs might be interesting.

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In conclusion, this work demonstrated that the LPS inserted in Cuban PLs has a dramatically reduced ability to induce expression of pro-inflammatory cytokines relative to commercially available purified LPS. The ability of the PLs-LPS to induce cytokine expression was restored after fractionation of the PLs, indicating that the low pro-inflammatory activity of PLs results from the insertion of LPS in the PLs rather than from a chemical modification of the LPS. These results could be useful in the use of PLs from different sources as immunogens and adjuvants. Finally, our results suggest the possibility that PL may contain pro-inflammatory components other than LPS or PorB. Identifying these components might shed light on the mechanisms by which PLs derived from an extra cellular bacterium induce a Th1 response.

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Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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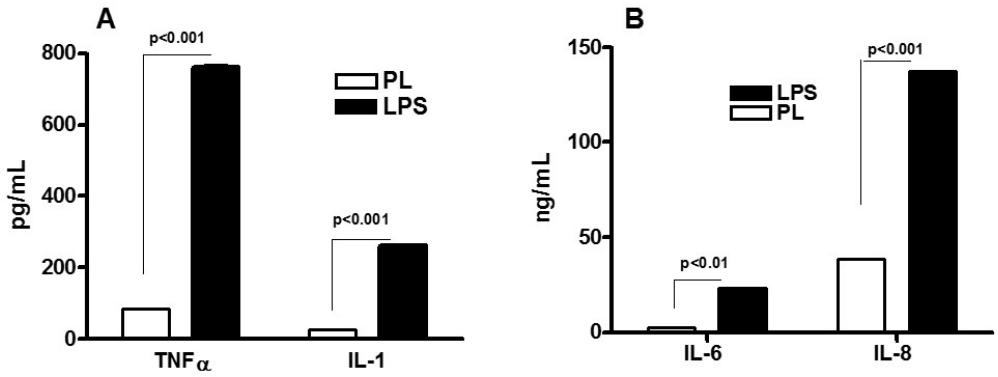

Fig 1

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Figure 1. LPS inserted in Proteoliposome (PLs) induced levels of cytokine response dramatically lowers than purified lipopolysaccharide (LPS). Differentiated human U937 histiocytic cell line was incubated with PL (250 ng/mL) or LPS (10 ng/mL). LPS concentration in the PL was 10 ng/mL. Fig. 1A shows TNFα and IL1β production (pg/mL) and Fig. 1B shows IL6 and IL8 production (ng/mL) 24 h after stimulation as determined by ELISA. Data are presented as mean cytokine concentration in supernatants ± standard deviation of al least three different experiments. Significant differences between the means of two different groups were determined by a Student t test using statistical analyses with Graph Pad Prism 4 software (Calif.) and a p-value of <0.05 was considered statistically significant.

Fig 2

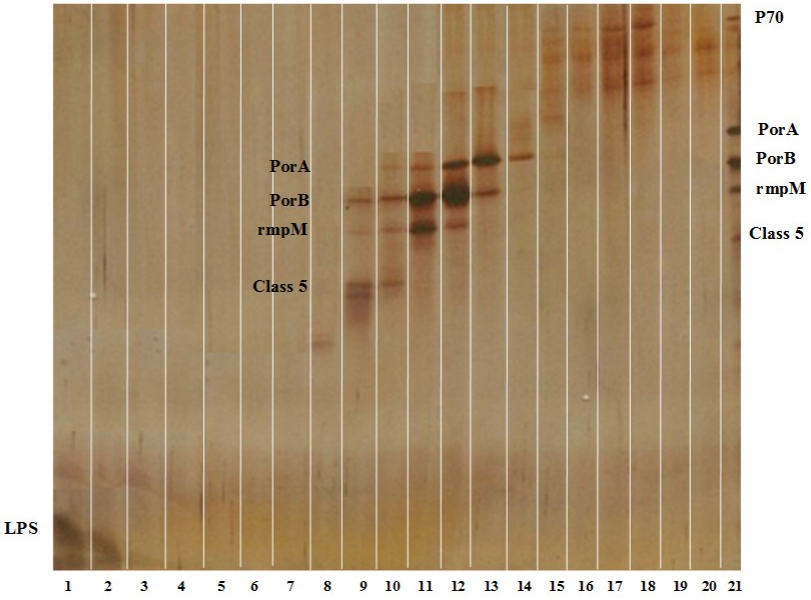

Figure 2. Twenty fractions were obtained by slot elution following SDS-PAGE separation of Proteoliposome (PL). A first SDS-PAGE analysis of PL was performed on 12% polyacrylamide gels and electroeluted. Then, the initial migration and the remaining proteins after electroelution were stained with Coomassiee to visualise proteins (not shown in this figure). A second SDS-PAGE was performed to determine the electroeluted proteins which was stained with Silver. High molecular

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weight standard was included in each gel. White lines were included to separate the fractions (1-20). Molecular weights were calculated with the molecular analyst program (Bio-Rad). Lipopolysaccharide (LPS) was clearly present in fractions 1 and 2, while the principal meningococcal outer membrane proteins where present in various fractions, but mainly in one of them and could be only tentatively identified at this stage. PL was included as control in line 21. Main proteins were also marked in the middle of the figure.

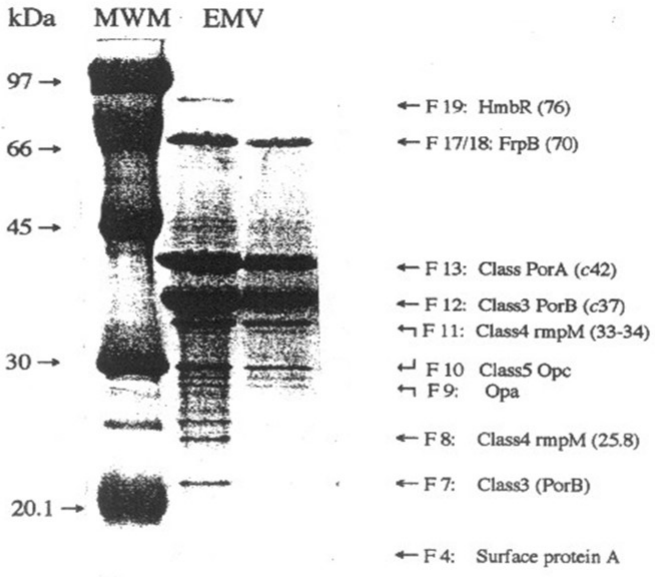

Fig 3

Figure 3. Eight meningococcal outer membrane proteins were identified in Proteoliposome (PL) fractions by end terminal sequencing. Gels at 12% with PL (external membrane vesicles, EMV) neat (1 mg/mL) appeared in the right column or concentrated 5 times (central colum) to better see the less represented proteins were performed. The proteins were transferred to nitro-cellulose, stained with Coomassiee and cut to sequence. The mains proteins from fractions 11-13 and 18 of Fig.

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2 were also used to verify that each band correspond to the PL proteins. The sequences were identified comparing with those of the data bank (www.ncbi.nlm.nih.gov).

Fig 4

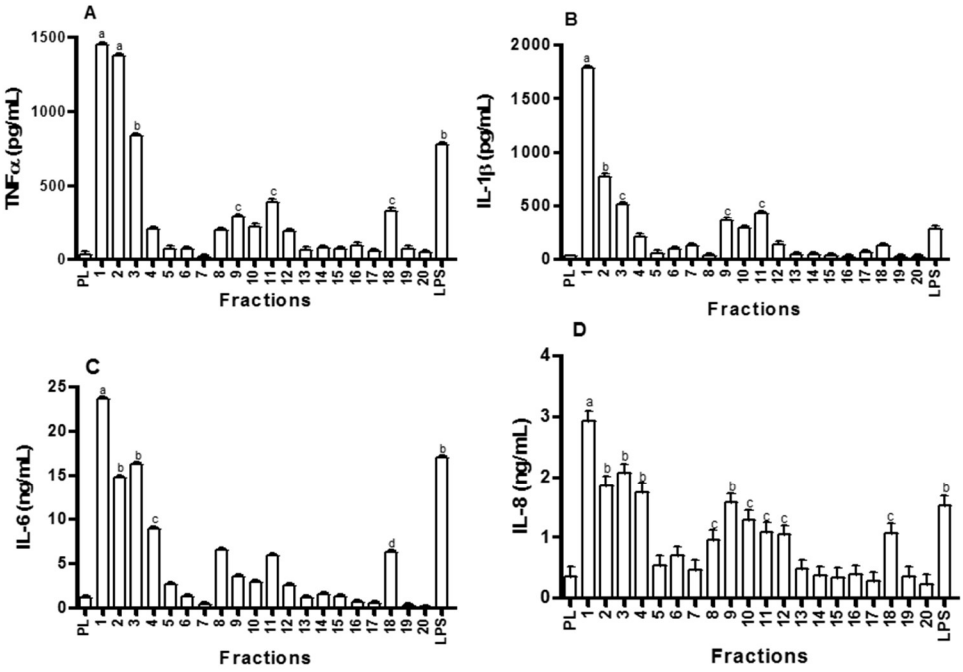

Figure 4. Several individual fractions induced dramatically higher levels of cytokine production than whole Proteoliposome (PL). The differentiated human U937 histiocytic cell line was activated with whole PL, each PL fraction, or LPS. TNFα and IL1β production (pg/mL) and IL-6 and IL-8 production (ng/mL) 24 hours after stimulation is showed as determined by ELISA. Data are presented as mean cytokine concentration in supernatants ± standard deviation less the background level of unstimulated cells of three different experiments. Significant differences between the means of different activated cell were determined by a Tukey multiple comparison test using the Graph Pad Prism 4 software (Calif.). A p-value of <0.05 was considered statistically significant and it is represent by different letters, a ($p<0.001$); b is ($p<0.01$); and c ($p<0.05$).

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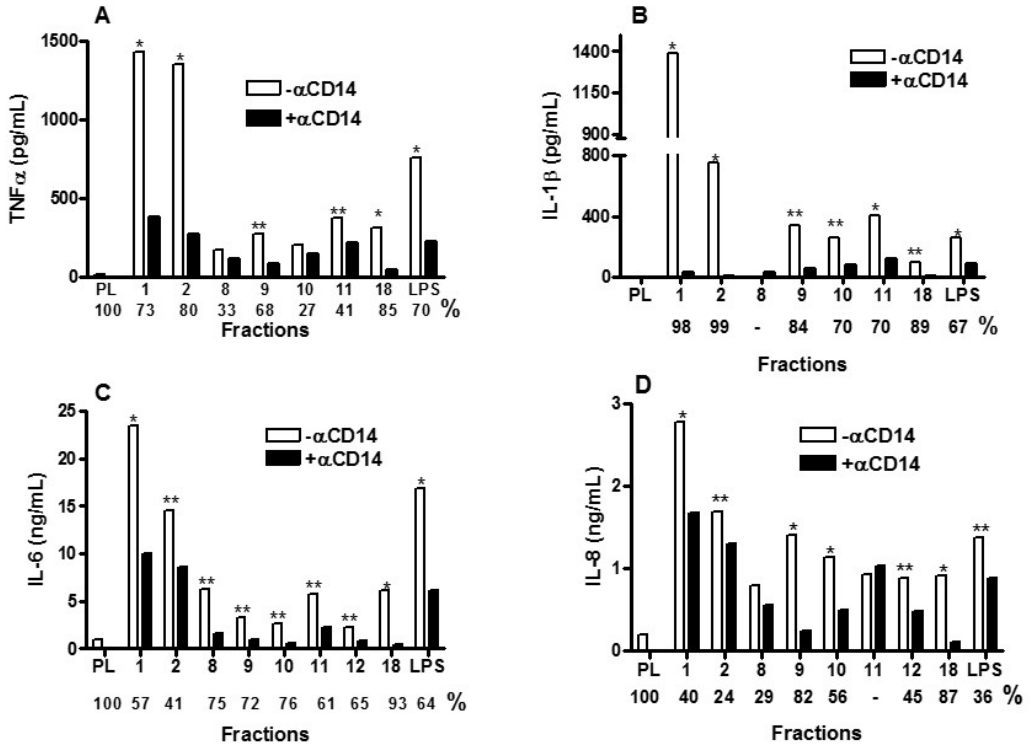

Fig 5

Figure 5. Pre-incubation of the cells with anti-CD14 mAb considerably reduced induction of cytokine production by most fractions but not inhibited IL-8 production by F11. The differentiated human U937 histiocytic cell line was activated with each PL fraction, whole PL or LPS. Parallel plates were treated with an anti mouse CD14 mAb before activation. TNFα and IL1β production (pg/mL) and IL6 and IL8 production (ng/mL) 24 h after stimulation is showed as determined by ELISA. Data are presented as mean cytokine concentration in supernatants ± standard deviation of three different experiments. Significant differences between the means of two different groups were determined by a Student t test using statistical analyses with Graph Pad Prism 4 software (Calif.). A p-value of <0.05 was considered statistically significant and it is represent by asterisk, * ($p<0.001$) and ** is ($p<0.01$).

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