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Vaccine 22 (2004) 3045–3052
Vaccine
www.elsevier.com/locate/vaccine

Very small size proteoliposomes derived from *Neisseria meningitidis*: an effective adjuvant for Th1 induction and dendritic cell activation

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Received 9 June 2003; accepted 5 February 2004

Available online 5 March 2004

Abstract

Recent findings about pathogens and innate immune system interactions have opened new opportunities for adjuvants designs. We have elaborated a new approach, in which gangliosides are incorporated into the outer membrane complex of *Neisseria meningitidis* (Nm) to form very small size proteoliposomes (VSSP). VSSP, used as monotherapy, demonstrated a unique ability to render immunogenic highly tolerated gangliosides. These results drove our attention to the immunopotentiating properties of VSSP. Here, we examined the VSSP adjuvant effect on the humoral and cellular responses, dendritic cell (DC) activation, and differentiation of Th cells. Also, the role of LPS in VSSP effect was dissected. This study reveals that VSSP is a potent adjuvant for dendritic cells activation and Th1 differentiation.

Keywords: Dendritic cells; Th1/Th2 cells; *Neisseria meningitidis*

1. Introduction

Adjuvants are important components of vaccine formulations, acting as enhancers of antigens' immunogenicity. However, traditional adjuvants sometimes do not stimulate protective immunity because they fail to fully mobilize the appropriate responses. Moreover, many of them promote serious adverse side effects and are therefore, not suitable for human use.

Emerging theories and knowledge about the immune system regulation have strongly influenced in adjuvant development. New pathogen-related molecules have been recently identified as "danger" signals [1], switching on the innate immune system, basically by dendritic cells (DC) maturation. It has also been proposed that the pattern recognition receptors expressed on DC interact with their ligands to subsequently condition either a Th1 or Th2 response [2,3].

Neisseria meningitidis (Nm) interacts with DC inducing strong secretion of proinflammatory cytokines TNF-α, IL-6, IL-8 and IL-12 production [4,5]. This bacteria spontaneously sheds natural outer membrane vesicles (nOMV) [6], which are considered potential adjuvants since many of their components are B-cell mitogens and others, such as the major antigen PorA, augment the allostimulatory properties of treated DC [7–9]. In this respect, we have described new cancer vaccines formulations based on nOMV adjuvanticity properties and the hydrophobic incorporation of NAcGM3, NGcGM3 or NAcGM1 gangliosides to form very small size proteoliposomes (VSSP) [10]. Immunization of mice with VSSP emulsified in an oily adjuvant consistently induced highly specific IgM and IgG antibodies against the incorporated ganglioside. Moreover, vaccination with $VSSP^{NAcGM3}$ increased the overall survival of mice challenged with the NAcGM3 positive melanoma B16 tumor [11]. Phase I trials of VSSP in melanoma and breast cancer patients have already demonstrated the safety and immunogenicity of these preparations [12].

This peculiar ability of VSSP to render immunogenic ubiquitous gangliosides, suggested strong immunopotentiatory properties for VSSP, in which incorporated gangliosides could play a role in its physicochemical characteristics. Also, a potential biological role of the ganglioside in VSSP should not be neglected and is currently under investigation.

In the present study, we established that VSSP promoted strong antibody and cellular responses to OVA, either emulsified or not. The adjuvanticity of this formulation is mediated by a proper DC maturation, with the corresponding IL-12p40/p70 production. Experiments with transgenic mice-derived T cells showed that VSSP conditioned a Th1 phenotype on stimulated naïve T cells. As VSSP are es-

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doi:10.1016/j.vaccine.2004.02.010

sentially bacterial membranes, we studied the contribution of LPS on VSSP properties. LPS hyporesponsive C3H/HeJ mice-derived DC were properly activated by VSSP.

2. Materials and methods

2.1. Mice

For in vivo experiments Balb/c mice were purchased from the Centre for Laboratory Animal Production (CENPALAB, Havana, Cuba) and maintained in the animal house of the CIM, Havana, Cuba. Balb/c (for in vitro experiments) and DO.11.10 αβ TCR transgenic (Tg) mice were bred and maintained in the SPF unit of the Institute for Animal Health, Compton, Berkshire, UK. C3H/HeJ and C57Bl/6 mice were supplied by Harlan UK, Bicester, Oxon. All animals were between 6 and 12 weeks of age.

2.2. Reagents used for stimulation studies

Dr. Svein Andersen, EJIVR, kindly provided purified lipopolysaccaride (LPS) from *N. meningitidis* strain 44/76. Monophosphoryl lipid-A (MPL-A) from *Escherichia coli* strain F583 was purchased from Sigma Chemical, UK, and the anti-mouse CD40 (1C10) from R&D Systems Ltd., Europe. VSSP [10] is produced and provided by the Centre of Molecular Immunology (Havana, Cuba).

2.3. Immunizations

For humoral response experiments, three groups of 10 Balb/c mice were immunized s.c., three times at 2-week intervals, with 50 μg of OVA (Sigma, San Louis, MO), emulsified by mixing equal volumes of OVA solution with complete Freund's adjuvant (CFA), or OVA + VSSP (120 μg) solution with Montanide ISA 51 (Seppic, France), a special type of incomplete Freund's adjuvant [13]. A non-oily immnunogen was also prepared by mixing 50 μg of OVA with 120 μg of VSSP in Tris–HCl buffer (pH 8.5). Animals were bled 1 week before and after the last immunization. Sera were stored at −20 °C.

For cellular response experiments, one single dose of 100 μg of OVA plus adjuvants were administered s.c. near the base of the tail, to three groups of Balb/c mice (three animals each). One week later, mice were killed and inguinal lymph nodes were removed.

2.4. ELISA assay for OVA-specific antibody production

Solid-phase ELISA was performed using 96-well polystyrene plates (High binding, Costar), coated with 10 μg/ml of OVA (Sigma, San Louis, MO) overnight at 4 °C. Then the diluted serum samples were incubated 2 h at 37 °C. The second antibody, Fc fragment-specific biotinylated rat anti-mouse IgG (Jackson, WestGrove, Pensylvania), was added and after 1 h of incubation the plates were washed, then streptavidine–phosphatase conjugate (Jackson, WestGrove, Pennsylvania) was added and the reaction developed with TMB substrate (PharMingen, San Diego, California, USA) until the addition of H_2SO_4 1N. Absorbance was measured at 405 nm in an ELISA reader (Organon Teknika, Salzburg, Austria).

2.5. Cellular proliferation assay

Inguinal lymph nodes from mice previously immunized with OVA, as described, were smashed and made into single cell suspension in RPMI 1640 (Gibco, UK) with Glutamax I and 25 mM HEPES (Gibco, UK) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. Obtained cells were then cultured in the presence of graded amounts of OVA. Proliferation was monitored by measuring [methyl $-^3$H] TdR (1 μCi per well) (Amersham, UK) incorporation on day 4 of culture. Radioactivity was determined on a Topcount microplate scintillation counter (Wallac, Finland).

2.6. FACS analysis

DC were analyzed for expression of surface molecules by flow cytometry. Cells were stained with either PE- or FITC-conjugated MAb: hamster anti-mouse CD11c (HL3) conjugated to PE, CD40 (HM40-3) FITC, rat anti-mouse MHC class II/I-Ed (2G9), CD80/B7.1 (GL1), CD86/B7.2 (GL1) all conjugated to FITC, and anti-CD4 (RM4-5) conjugated to allophycocyanin (APC) for CD4 T cell sorting. For human experiments mouse antihuman CD86 (IT2.2), HLA-DR (G46-6), CD1a (HI149), CD11c (B-ly6) conjugated to PE and anti-CD40 conjugated to FITC, were used. Intracellular detection of IL-12 was performed using BD Cytofix/Cytoperm™ Plus kit with BD GolgiPlug™ kit following the protocol recommended by the manufacturer and using the APC-conjugated rat anti-mouse IL-12p40/70 (C15.6). Permeabilization kit, antibodies and all of the matching isotype controls were from Pharmingen, Becton Dickinson, UK.

2.7. Mouse bone marrow DC preparation

Bone marrow (bm)-derived DC were prepared as described elsewhere [14]. Briefly, bone marrow cells were cultured in Isocove's Modified Dulbecco's medium (Life Technologies Ltd., Paisley) supplemented with 10 ng/ml of GM-CSF (R&D Systems, Abingdon, Oxon) at approximately 10^6 cells/ml. At day 7, bmDC were further purified (>95%) using magnetic anti-CD11c beads, according to the manufacturer's instructions (Milteny Biotec, Surrey, UK). Following isolation, bmDC were resuspended in fresh media at 10^6 cells/ml with or without additional stimuli.

2.8. Human DC preparation

PBMC were isolated by standard density gradient centrifugation with Histopaque (1.077 g/ml) (Sigma, UK). PBMC were harvested for the subsequent monocyte isolation. $CD14^+$ cells were enriched by high gradient magnetic sorting negative selection using the MidiMACS monocyte isolation kit (Miltenyi Biotech GmBH, Germany) following supplier's recommendations. Isolated $CD14^+$ monocytes were cultured in RPMI (Life Technologies Ltd., Paisley) supplemented with 50 ng/ml of recombinant human (rh) GM-CSF and 1000 U/ml of rh IL-4 (R&D Systems, UK). At day 7 cells were cultured with or without additional stimuli.

2.9. Antigen-specific T cell activation assay

Purified T cells from DO.11.10 mice were used as source of antigen-specific responder cells. The naive cells were first isolated from spleens using anti-mouse CD62L magnetic beads (Milteny Biotec, Germany) and further stained with APC-conjugated CD4 for sorting by MoFlo FACS sorter (Cytomation, Ely, UK). T cells were then cultured with the relevant bmDC and different concentrations of Ovalbumin peptide (323-ISQAVHAAHAEINEAGR-339) (Genosys Europe, Pampisford, Cambridgeshire, UK). Proliferation was monitored by measuring [methyl $-^3$H] TdR (1 μCi per well) (Amersham, UK) incorporation on day 4 of culture. For the cytokine assays, at day 3 of culture, 500 ng/ml ionomycin and 50 ng/ml PMA (Sigma, San Louis, MO) were added to the cells. Supernatants were removed 24 h later and cytokines were evaluated using QuantikineTM Mouse ELISA kits (R&D Systems Ltd., Abingdon, UK), following the protocol recommended by the manufacturer.

2.10. RNase protection assay

Bone marrow DC from C57Bl/6 and C3H/HeJ mice were cultured in the presence of different stimuli for 8 h. The total RNA was extracted using RNeasy Midi kit (Qiagen Ltd., UK). Cytokine transcript levels were determined by RPA using RiboQuantTM kits (Pharmingen, Becton Dickinson, UK) with the probe sets mCK-5 and mCK-2b. The intensities for each band were corrected for background levels and normalized for differences between each sample using the average values for the housekeeper genes GAPDH and L32.

2.11. Statistical analysis

Differences in humoral response between all treatment groups were analyzed by one-way ANOVA and Tukey's test for pairwise comparisons. For cellular response the significance of differences between all experimental groups was analyzed by Kruskall Wallis and Tukey's test for pairwise comparisons. Value were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. VSSP promote antigen-specific humoral and cellular responses

We have previously shown, in the ganglioside model, that immunization of mice with VSSP/Montanide ISA 51 induced high levels of anti-ganglioside-specific IgG isotypes [10]. However, the VSSP adjuvant effect for an accompanying protein has never been assessed, nor the mandatory need of emulsions. To address this point, groups of Balb/c mice were injected s.c. with OVA in the presence of VSSP, either emulsified or not in Montanide ISA 51, the same preparation with CFA, as a reference, was used. As shown in Fig. 1a, emulsified VSSP induced similar anti-OVA IgG levels compared with CFA (Tukey's test, $P = 0.151$). Moreover, VSSP alone produced large quantities of IgG, reaching antibody titters up to $1/6 \times 10^6$. Anti-OVA cellular

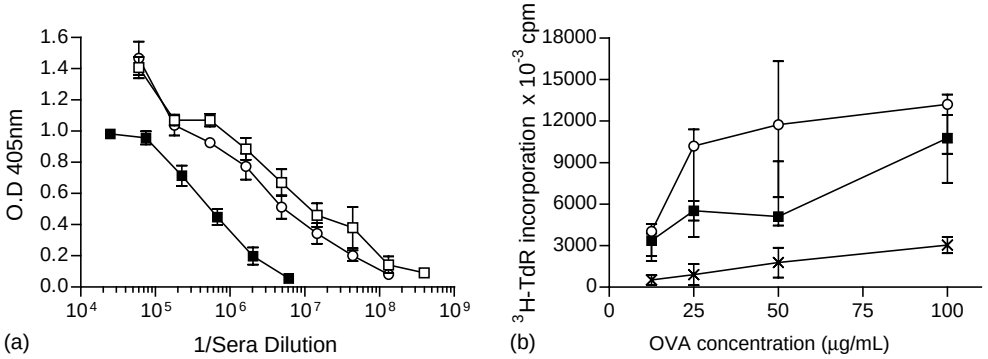

Fig. 1. Antigen-specific humoral and cellular responses induced by immunization of Balb/c mice with OVA/VSSP. (a) Humoral response. Three groups of 10 mice were injected s.c. with OVA/CFA (○), OVA/VSSP/Montanide ISA 51 (□) or OVA/VSSP (■). Sera were collected from mice 7 days after the last injection and assayed by ELISA for anti-OVA total IgG. Data are represented as mean ± S.D. (b) Cellular response. Cells from drained lymph nodes were obtained 1 week after a single dose of OVA/VSSP (■) or OVA/CFA (○) or OVA/PBS (×), to perform a cellular proliferation assay. Median and ranges, corresponding to three mice from each group of immunization, are shown.

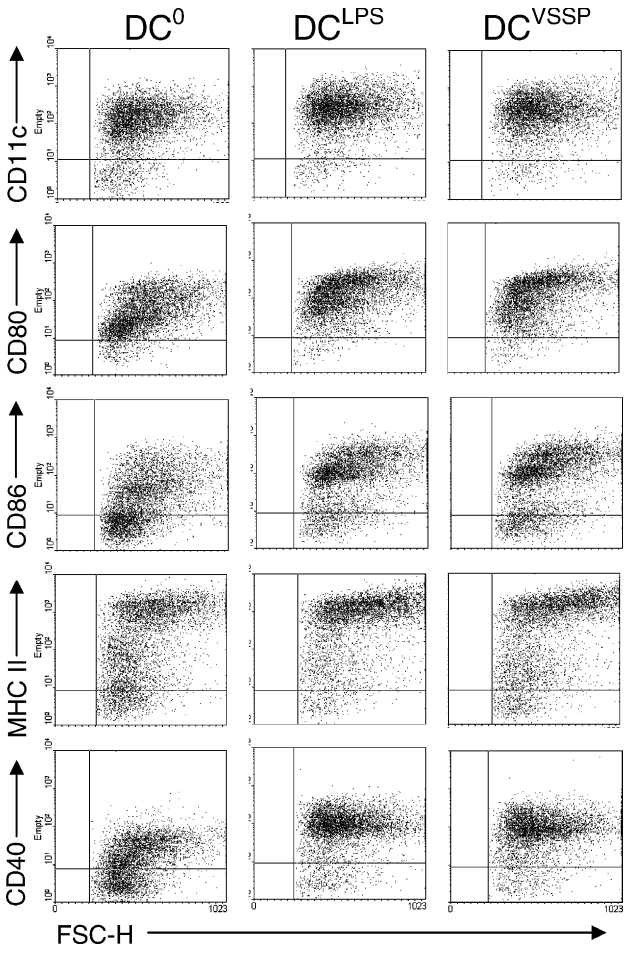

Fig. 2. Dendritic cell maturation induced by VSSP. CD11c$^+$ bmDC were cultured for 24 h with VSSP, LPS or medium and expression of CD11c, MHC II, CD80, CD86 and CD40 was examined by flow cytometry. Data are presented in dot plots from a single experiment representative of several others.

response elicited by non-emulsified VSSP was also measured in a proliferation assay. Different amounts of OVA were added to inguinal lymph nodes cells obtained from mice immunized with a single OVA-VSSP dose. CFA was again used as a reference. Noteworthy, VSSP was able to induce an OVA-specific proliferative response similar to that induced by CFA (Tukey's test, $P = 0.185$) (Fig. 1b).

3.2. VSSP induce maturation of bmDC

Dendritic cells are unique professional APC that play critical roles immune responses [16]. Thus, we evaluated the effect of VSSP in DC expression of class II MHC and a variety of co-stimulatory molecules. Fig. 2 clearly shows that VSSP-treated DC (10 μg/ml) up-regulated CD80, CD86, CD40 and MHC II. Importantly, LPS (1 μg/ml) was indistinguishable from VSSP for inducing similar expression of these molecules.

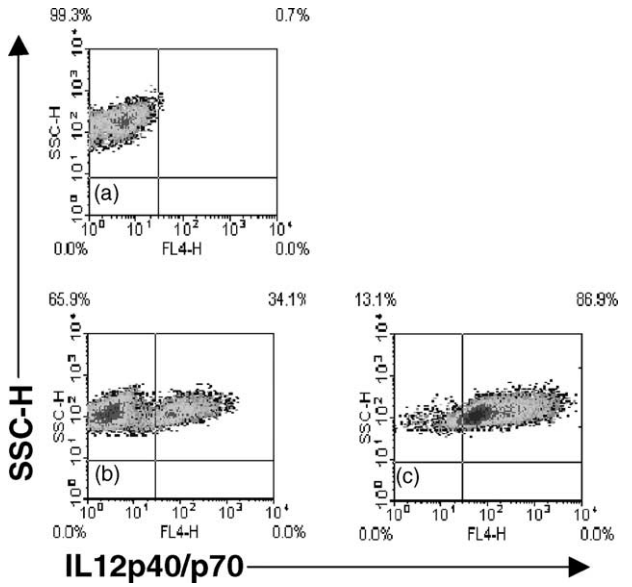

Fig. 3. IL-12p40/p70 production induced in bmDC by VSSP. Bone marrow-derived DC were stimulated with either medium (a), LPS (b) or VSSP (c) in the presence of Golgi Plug (Brefeldin A). Intracellular cytokine production was assessed after 24 h. Data are presented in density dot plots from a single experiment representative of four experiments yielding comparable results.

3.3. VSSP promote IL-12p40/p70 production

To evaluate the capacity of VSSP to induce IL-12 production, the intracellular cytokine levels in the gated dendritic cell population were measured by flow cytometry. Differences between LPS- and VSSP-treated DC were observed. Although a high percentage (87%) of IL-12p40/p70 producing DC were detected after stimulating with VSSP, only 34% of LPS stimulated DC produced this cytokine (Fig. 3).

3.4. VSSP induce activation of bmDC derived from LPS hyporesponsive mice

To investigate the possibility that components of VSSP other than LPS could induce DC maturation, we first determined the surface markers expression on LPS hyporesponsive C3H/HeJ mice-derived bmDC after culture with LPS (1 μg/ml), VSSP (10 μg/ml), medium alone or with an anti-mouse CD40 MAb (2 μg/ml) as a positive control [15]. As expected, the effect of LPS was weak as indicated by the low expression of MHC II, CD80, CD86 and CD40. Strikingly, VSSP induced the expression of these markers (Fig. 4).

We also tested if other components of VSSP, in addition to LPS, could promote DC activation. For this purpose, VSSP- and LPS-conditioned C3H/HeJ-derived bmDC cytokine and chemokine profiles were examined. Results from the RNA protection assay (Fig. 5) showed that both LPS and VSSP induced similar cytokines and chemokines pattern in

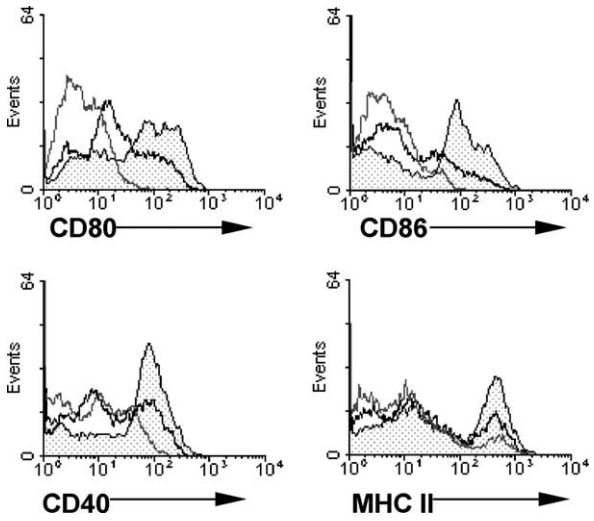

Fig. 4. Maturation of DC derived from the LPS hyporesponsive mice C3H/HeJ by VSSP. C3H/HeJ-derived CD11c$^+$ bmDC were cultured for 24 h with VSSP (light gray solid), LPS (black line), or medium (gray line) and expression of MHC II, CD40, CD80 and CD86 was examined by flow cytometry. Data are presented as histograms from a single experiment representative of three others.

bmDC from C57Bl/6 mice, while bmDC from C3H/HeJ remained hyporesponsive to LPS. In contrast, whilst only mRNA for IL-1α was absent, the rest of the induced genes in C57Bl/6 DC were also detectable on C3H/HeJ-derived DC in response to VSSP. In particular, IL-12p40, IL-6

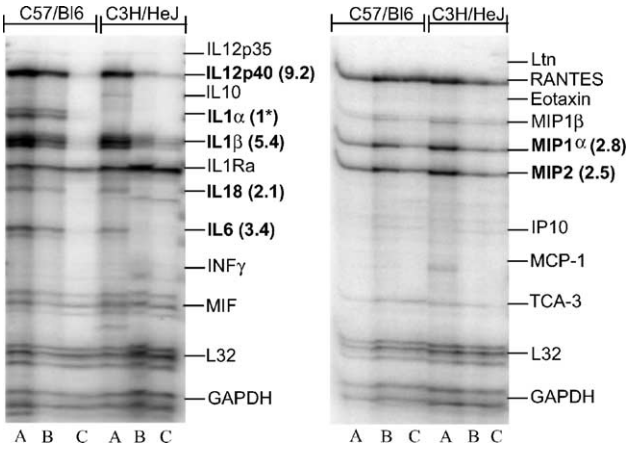

Fig. 5. Inflammatory chemokines and cytokines mRNA induced by VSSP in DC derived from normal and LPS hyporesponsive mice. CD11c$^+$ bmDC derived from C3H/HeJ or C57Bl/6 mice were incubated with VSSP (A), LPS (B) or medium (C) for 8 h and an RNAse protection assay was performed to determine induction of the indicated cytokines and chemokines at the mRNA level. In bold are those proteins for which the gene transcription was induced in C57Bl/6 DC ($DC^{AorB}/DC^{C} \geq 2$; after normalization). Numbers in brackets indicate the relative gene transcription induced by VSSP in C3H/HeJ DC given by the ratio of DC^{A}/DC^{C}, after normalization. For the rest of the evaluated cytokines and chemokines there was no induction of transcription ($DC^{A-B}/DC^{C} = 1$) in both mouse strains.

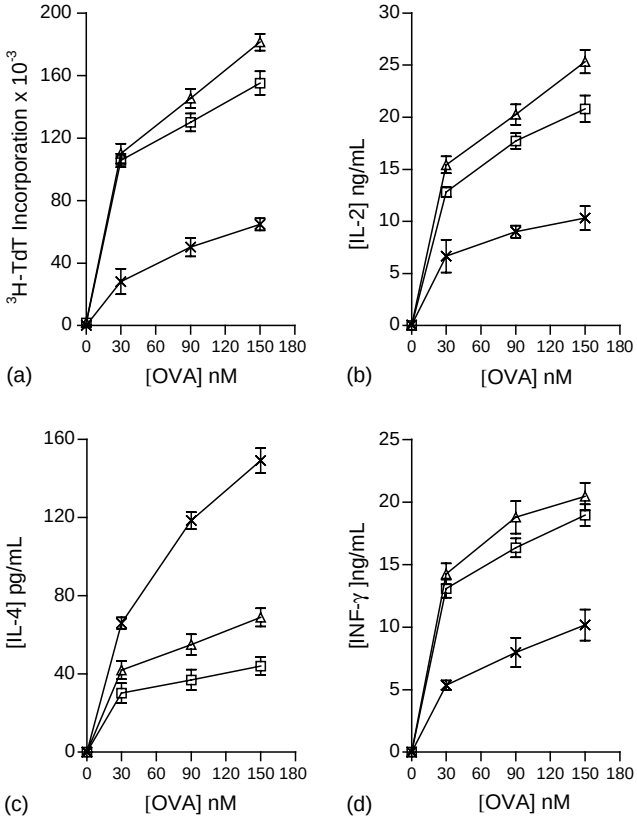

Fig. 6. Th1-associated OVA-specific responses promoted in naïve CD4$^+$ T cells by VSSP-treated DC. bmDC matured in the presence of VSSP (□), LPS (△) or media (×) were co-cultured with DO.11.10 Tg naive CD4$^+$ T cells at the indicated OVA concentrations. Figure shows: (a) DNA synthesis and (b) IL-2, (c) IL-4 or (d) INF-γ production. Data are presented as means ± S.D. from a single experiment representative of at least three other independent experiments.

and IL-1β were seen at high levels suggesting that VSSP contains components, in addition to LPS, that signal DC (Fig. 5).

3.5. VSSP promote an OVA-specific Th1 response on naïve T cells

Once we knew that VSSP can stimulate DC, the capacity of VSSP-treated DC to activate and drive antigen-specific Th1/Th2 responses on naïve T cells was studied. CD4$^+$ T cells purified from DO.11.10 mice were incubated with bmDC from each stimuli condition. VSSP- or LPS-matured bmDC revealed a DC phenotype that promoted increased proliferation of CD4$^+$/CD62^{high} Ag-specific Tg T cells at all OVA concentrations tested (Fig. 6a). To determine whether the observed increase in proliferation was associated with either Th1 or Th2 cytokine profiles, the secretion of IL-2, INFγ and IL-4 were evaluated. VSSP-treated bmDC promoted a Th1 profile of cytokine secretion in CD4$^+$ T cells (Fig 6b–d), characterized by an increased INFγ production and inhibited IL4 secretion.

Table 1
VSSP induces human DC maturation

Treatment	CD11c	CD83	CD86	CD40	HLA-DR
DC^0	36.7	2.0	2.7	2.6	35.0
DC^{LPS}	160.2	5.6	8.5	5.0	92.4
DC^{MPLA-A}	70.7	2.8	4.1	2.8	71.6
DC^{VSSP}	198.5	6.5	10.1	4.5	104.2

Data are representative of one of the three experiments expressed as MFI values calculated after the MFI values obtained with isotype control Mabs were subtracted.

3.6. VSSP is more effective than MPL-A in the activation of human DC

MPL-A is a derivative of LPS that retains most of its endotoxin bioactivities [17], but with attenuated toxicity [18,19]. It has been administered safely to humans in various clinical trials [20], so we compared the potency of VSSP relative to MPL-A on human DC activation. Monocyte-derived immature DC were cultured for 24 h in the presence of VSSP (1 μg/ml), LPS (0.1 μg/ml), MPL-A (1 μg/ml) or medium alone and the surface expression of several DC maturation markers was measured by FACS. DC treated with MPL-A (DC^{MPL-A}) had higher expression of CD11c, CD83, CD86 and HLA-DR than medium-treated DC (DC^0). However, the levels of these molecules, also including CD40, on DC^{VSSP} and DC^{LPS} were significantly greater than those of DC^{MPL-A} (Table 1).

4. Discussion

Previously, we have demonstrated that immunization with emulsified VSSP consistently induced IgM and IgG antibodies against highly tolerated gangliosides [10,11]. To define the advantage of using this new adjuvant in future vaccines OVA was mixed with VSSP emulsified in Montanide ISA 51 (IFA), VSSP alone or CFA (as a reference adjuvant) and compared the anti-OVA antibody titters elicited in the immunized mice. The same intensity in the antibody immune responses was observed using CFA and IFA/VSSP. A common feature of these adjuvants is that they are both formulated with bacterial products (*Mycobacterium tuberculosis* (Mt) and Nm for CFA and VSSP, respectively) and emulsified in incomplete Freund's adjuvant (IFA). This result suggests that VSSP could be a safer alternative of CFA that can be used in therapeutic vaccines for humans, at least for the induction of antibody responses. More interestingly, this experiment also shows that VSSP alone can be used as an adjuvant. The antibody titters induced in mice immunized with OVA/VSSP alone were significantly lower than those measured in the corresponding animals inoculated with OVA/emulsified VSSP. Nevertheless, the specific antibody levels induced in OVA/VSSP-treated animals were notoriously high (1.6×10^6). Moreover, VSSP has also shown its ability to stimulate OVA-specific T cell responses. Proliferation of spleen cells obtained from mice after one single dose of OVA formulated with CFA or OVA co-administered with VSSP were not significantly different. A number of bacterial products which are effective immunostimulants [2,3] have been reported. We demonstrated that VSSP belongs to this privileged group.

The fact that VSSP alone can stimulate the anti-OVA immune response means that we can probably eliminate the oily component-associated toxicity of emulsified vaccine formulations. Another very important advantage of VSSP is its own capability to exert an adjuvant effect without any covalent conjugation to the nominal antigen.

The discovery and characterization of adjuvants that promote a Th1 cell-mediated immunity is currently an important area in vaccine development. Our studies also addressed the ability of VSSP to modulate the differentiation of Th responses. Different subsets of DC act as a bridge between pathogens and T cells [21]. In this way, myeloid DC preferably induce Th1 differentiation while lymphoid DC induce Th2 [22]. However, a certain degree of flexibility on the ability of each DC subset in directing T cells responses has also been documented. This depends on signals from pathogens and the microenvironment [23]. Based on this "functional plasticity" of DC we have studied the roll of these cells on the Th1 response triggered by VSSP. Thus, firstly, we measured the capacity of VSSP to mature DC. Stimulation of bmDC for only 18 h with VSSP leads to increased expression of MHC II and co-stimulatory molecules compared with non-stimulated DC. This result was also obtained with human DC derived from monocytes. Interestingly, no differences on DC activation were found between VSSP and LPS. Due to its marked toxic side effects, LPS are not suitable for use in vaccines. On the other hand, certain adjuvants have been formulated using MPL-A, which has been reported to retain the immuno-stimulating activities of the parent LPS molecule [18], but has high attenuated toxicity [19]. Some of these adjuvants have been tested in animals models and clinical trials showing an acceptable profile of toxicity and efficacy [20]. Curiously, in this study, we also established that VSSP has a more powerful effect over human DC than MPL-A.

Results of the in vitro T helper cell assay, in which naïve CD4+ T cells from DO.11.10 Tg mouse were cultured with VSSP-matured bmDC in the presence of OVA, illustrated how VSSP preferentially stimulated T cells to secrete INFγ whilst IL-4 production was diminished. This model has been previously used to demonstrate opposite effects for LPS and a nematode-derived protein [24], validating the potent Th1 promoter effect of VSSP.

IL-12 produced by DC cells is pivotal for the development of Th1 responses [25]. The intracellular staining experiments showed a high level IL-12 production by DC in response to VSSP. IL-12 production by DC has been documented for

many gram-negative bacterial products like OmpA [2] and CpG [26]. Recently, the secretion of IL-12 by DC in response to *N. meningitidis* has been also reported evidencing that this phenomenon requires LPS expressed in the intact membrane of the bacteria [5]. However, the LPS requirement was questioned since the results from the RNase protection assay (RPA) showed that these nanoparticules are able to induce the transcription of the gene coding for the IL-12p40 (the inducible subunit of the active IL-12p70 [27]) in C3H/HeJ mice. In this experiment we also detected the induction of the IL-18 transcript on DC cultured with VSSP. IL-18 is a controversial cytokine regarding its link with Th1 or Th2 differentiation, but it is widely accepted that IL-18 acts in synergy with IL-12 to stimulate T cells to produce INFγ [28]. Another cytokine that came up with the RPA on VSSP-treated DC is IL-6. This constitutes an important finding since it has been recently reported that production of this cytokine by DC in response to TLR ligation during infection is critical for T cell activation. It renders antigen-specific T cells, refractory to the suppressive activity of $CD4^+CD25^+$ regulatory T cells [29].

In addition, to asses the role of LPS in VSSP, we studied the phenotype of VSSP-stimulated DC derived from the TLR4 (the transducing receptor for LPS [30]) mutant mice C3H/HeJ. As expected, DC maturation markers up-regulation, observed in normal mice after exposure to LPS, was abrogated in C3H/HeJ mice. Strikingly, VSSP was still able to induce normal levels of C3H/HeJ-DC maturation under the same conditions. In terms of DC cytokine and chemokine expressions in stimulated DC VSSP and LPS induced the same profile in normal mice while in C3H/HeJ mice a little up-regulation of some cytokines with LPS and a normal response with VSSP were observed. These data suggest that VSSP could additionally be engaging other receptors different from TLR4 to induce DC activation. The TLR4 involvement was evidenced by the lack of IL-1α up-regulation in C3H/HeJ DC after either VSSP or LPS exposure. These results may indicate that VSSP is composed of a variety of pathogen-associated molecular patterns (PAMP) capable of interacting with TLR4 and with at least one more pattern recognition receptor (PRR) on DC. This in addition to the complexity of VSSP, can be considered as an advantage because the repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between TLRs. It has been reported that when TLR2 and TLR6 are recruited together to the phagosome they interact with peptidoglycan and induce cytokine induction. By contrast, TLR2 recognizes another component without TLR6 [31].

In this paper, we propose that VSSP acts as danger signals that warns of *N. meningitidis* infection and activates immune defenses. These danger signals provided by VSSP may induce potent adjuvant function through DC activation and Th1 polarization. Therefore, VSSP could be a good alternative to the existing adjuvants for use in future vaccines, a concept that is currently under investigation.

Acknowledgements

We would like to thank Professor Peter Beverley for his valuable suggestions. We are also grateful to Dr. Svein Andersen for providing LPS from *Neisseria meningitidis*, Drew Worth for FACS sorting, Dariel Morales for animal's care and handling, Marilyn Clavel for excellent technical assistance and to Dr. Blanca Tormo for her careful English revision. This work was supported by the Edward Jenner Institute for Vaccine Research (Compton, UK) and the Centre of Molecular Immunology (Havana, Cuba).

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