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Innate-immunity cytokines induced by very small size proteoliposomes, a Neisseria-derived immunological adjuvant

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Summary

Neisserial outer membrane proteins have been combined with monosialoganglioside GM3 to form very small size proteoliposomes (VSSP), a nanoparticulated formulation used as a cancer vaccine for the treatment of cancer patients with GM3-positive tumours. VSSP were shown to elicit anti-GM3 and anti-tumour immune responses. VSSP have also been shown to be an efficient adjuvant for tumour-cell and peptide-antigen vaccines in mice. *In vitro* studies showed that VSSP promote maturation of both murine and human dendritic cells, suggesting that VSSP could be used as efficient adjuvants. In order to study further the capacity of VSSP to elicit innate immune responses, human peripheral blood mononuclear cells and monocytes derived thereof were assessed for *in vitro* secretion of interleukin (IL)-10, IL-6, IL-12 and interferon (IFN)-γ. VSSP most prominently induced the secretion of IL-6. IL-10 was secreted at a lower level. IL-12 p40 (but no p70) was also detected. IFN-γ response was observed in 56% of the tested samples. Cytokine secretion was not related to lipopolysaccharide (LPS) content and involved Toll-like receptor 2 (TLR2)-mediated signal transduction. VSSP also induced DC maturation and a cytokine secretion pattern (high IL-6/low IL-10) which differs from that induced by LPS. The observed proinflammatory cytokine secretion pattern and the capacity of VSSP to drive DC maturation are examined in the light of the properties of other bacterial derivatives currently being user for immunotherapy purposes. Our results suggest that VSSP could be tested in clinical settings where T helper 1-type immune responses would be beneficial.

Keywords: adjuvants, cytokines, dendritic cells, immunotherapy, Neisseria

Introduction

GM3 is a tumour-associated antigen of enhanced expression in breast cancer and melanoma, and constitutes a potential target for immunotherapy [1]. The drawback for this monosialoganglioside is its very poor immunogenicity. This can be attributed to three main reasons: its glycolipid nature, its widespread cell surface expression in normal tissues and its being the most abundant ganglioside in serum [2]. The search for an immunologically active vaccine formulation led to the combination of GM3 with the outer membrane protein complex of *Neisseria meningitidis* to yield very small size proteoliposomes (VSSP). When used as an immunogen emulsified with an oil-based adjuvant, VSSP induced high anti-GM3 antibody titres (both IgM and IgG) in mice, chickens and monkeys [3]. Most interestingly, VSSP administration was capable of protecting against melanoma, as shown in C57Bl/6 mice with the B16 melanoma model [4,5].

These results, together with its favourable toxicity profile, prompted the assessment of its safety and immunogenicity in melanoma patients [6]. GM3-specific antibodies were induced in 44% of the patients and one of eight evaluated patients displayed an *in vitro* interferon (IFN)-γ response to GM3 as assessed by enzyme-linked immunospot assay (ELISPOT) [6]. Investigation of the contribution of the bacterial carrier to the immune response showed that it induced a strong IFN-γ response [6].

Whereas antigen and adjuvant functions have been assigned classically to different components of vaccine formulations, recent developments have led to the identification of a number of bacterial antigens that are themselves

strong immunoactivators and therefore do not require the use of further adjuvants. This opened the way for the development of novel immunological adjuvants. The need for T helper 1 (Th1)-inducing adjuvants, particularly in cancer immunotherapy, is underscored by the fact that aluminium-based adjuvants, in widespread clinical use, generate Th2-type responses [7].

Adjuvants chosen for experimental cancer immunotherapy [8] include bacterial derivatives such as bacille Calmette–Guérin (BCG) [9], OK-432 [10,11] and unmethylated cytosine-guanine motifs–oligodeoxynucleotides (CpG-ODN) [12], and they were all shown to promote Th1 responses. Essential to proinflammatory responses are the 'danger signals' provided by many of the above-mentioned adjuvants to dendritic cells (DC), which are instrumental to the effectiveness of the ensuing adaptive response [13].

Similar immunostimulating properties have also been found in isolated bacterial cell wall components. OK-432 activity has been recovered in a lipoteichoic acid-related molecule, named OK-PSA [14]. Outer membrane protein A (OmpA), purified from *Klebsiella pneumoniae*, has been shown to induce DC maturation and IL-12 secretion [15]. *In vivo*, OmpA mediates DC migration to the regional lymph nodes [16]. The potential use of such bacterial derivatives as immunological adjuvants remains to be investigated.

Neisserial proteoliposomes comprise a variety of cell-wall components. These include porins, which are capable of interacting with antigen-presenting cells via TLR2 and enhancing the expression of co-stimulatory molecules such as CD86 [17]. Proteoliposomes also contain lipopolysaccharide (LPS) (2–6% of protein content). LPS is a potent inducer of inflammatory cytokines and plays a major role in the development of septic shock [18]. As it raises the risk for undesired toxicity, LPS has been used only seldom in immunotherapy [19].

Studies with murine models have shown that VSSP is an effective adjuvant for tumour-cell and peptide-antigen vaccines [20]. VSSP induces murine dendritic cell maturation and cytokine secretion [21]. VSSP was also shown to increase the expression of maturation markers in human DC [21].

The purpose of the present work is to assess the *in vitro* VSSP-induced secretion of human monocyte and DC cytokines, and to determine the relative contribution of VSSP-contained LPS to its adjuvant activity.

Materials and methods

Peripheral blood mononuclear cells (PBMC) isolation and fractionation

PBMC were obtained from healthy volunteers after the approval by the Institutional Review Board (IRB) at the Hospital de Clinicas and with the donors' informed consent. PBMC were purified from heparinized blood (5–10 cc) by density gradient centrifugation (Histopaque 1077, Sigma Chemical Co., St Louis, MO, USA) and resuspended in Iscove's modified Eagle's medium (IMDM, Sigma) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 μg/ml gentamicin, 1 mM sodium pyruvate and non-essential amino acids (culture medium).

Monocyte isolation was performed by plastic adherence to the culture microwell plates. PBMC were seeded at 10^5 cells in 100 μl per well in round-bottomed 96-well plates and incubated for 2 h at 37°C in a humidified 5% CO_2 atmosphere. Non-adherent cells were gently resuspended and 60 μl were transferred to adjacent clean wells for recovery of non-adherent cells. Fresh culture medium was replaced (60 μl) in the original wells and the resuspension/transfer procedure was repeated twice. Finally, stimuli or plain culture medium was added to both the wells with adherent and non-adherent cells to a final volume of 200 μl for cell culture. As a indication of efficient cell separation, no IFN-γ secretion was detected in wells with adherent cells cultured with IL-2. Conversely, less than 10% of the LPS-induced IL-10 secretion was recovered in the non-adherent cell fraction.

PBMC samples were depleted of T lymphocytes following an indirect procedure with goat anti-mouse IgG magnetic beads (Dynal Biotech, Oslo, Norway). PBMC were incubated with affinity-purified monoclonal antibodies anti-CD4 (OKT-4 hybridoma; ATCC, Manassas, MD, USA) and anti-CD8 (UCHT-4 hybridoma). The monoclonal antibody (MoAb)-labelled cells were removed with the magnetic beads following the provider's instructions. The cells were resuspended in culture medium, and plated for cell culture at 10^5 cells per well as described below.

Natural killer (NK) cells were isolated negatively from PBMC cells using red blood cells coated with antibodies directed to CD3, CD4, CD19, CD36, CD66b and glycophorin A (Stemcell Technologies, Vancouver, BC, Canada) to yield 95% $CD53^+$ $CD16^+$ cells. Isolated NK cells were plated at $0{\cdot}5 \times 10^5$ cells per well.

Cell lines

THP-1 promonocytic leukaemia cells (ATCC) were maintained in culture medium and differentiated with the addition of 0·05 μM dihydroxyvitamin D_3 (Sigma) for 48 h prior to assay.

NK-L and NK-92 (ATCC) cell lines were maintained in culture medium supplemented with 100 U/ml IL-2. Cells were washed and resuspended in culture medium without IL-2 18 h prior to assay.

Cell culture

PBMC (10^5) (or the adherent or non-adherent cells obtained thereof) were cultured in triplicate wells in round-bottomed 96-well plates in a final volume of 200 μl. Where indicated, T cell-depleted PBMC were used at 10^5 cells per well. VSSP, neisserial OMP and GM3-loaded liposomes were obtained

from the Center of Molecular Immunology, Havana, Cuba. VSSP was produced by conjugating OMP hydrophobically from *N. meningitidis* strain 385 with GM3 ganglioside purified from canine red blood cells [3]. Unless specified otherwise, VSSP was added to the cell cultures to a final concentration of 5 μg ganglioside/ml (6·7 μg protein/ml). OMP concentration was 5 μg/ml and GM3-loaded liposomes [22] were added at 5 μg ganglioside/ml. Supernatants were recovered after 72 h incubation for cytokine quantification.

Isolated NK cells and NK cell lines were plated at $0{\cdot}5 \times 10^5$ cells per well in culture medium with the addition of any of the following: VSSP, polyinosinic–polycytdylic acid [poly(I:C)] (50 μg/ml) or IL-2 (100 U/ml). Supernatants were collected after 24 and 72 h incubation for IFN-γ quantification.

Immature DC were obtained by culture of adherent monocytes for 6 days in the presence of IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF) in six-well plates at an initial density of $2{\cdot}5 \times 10^6$ PBMC per well. DC maturation was promoted by the addition of 5 μg/ml GM3/VSSP, 1 μg/ml LPS or 50 μg/ml poly(I:C) for another 48 h. At that time, phase contrast pictures were taken with a Nikon Labophot microscope, supernatants were saved for cytokine quantification and cells were harvested for assessment of CD80 and CD86 expression.

Anti-TLR antibodies

PBMC were plated at 10^5 cells per well in 96-well, round-bottomed plates in 100 μl. TLR4 was blocked with 20 μg/ml HTA-125 MoAb (eBioscience, San Diego, CA, USA) for 1 h at 37°C. VSSP (1 μg ganglioside/ml), LPS (0·05 μg/ml) or plain culture medium were then added to a final volume of 200 μl. Supernatants were collected after 18 h incubation for IL-6 quantification.

Differentiated THP-1 cells were plated at 2×10^4 per well in 100 μl. TLR2 was blocked with 20 μg/ml TL2·1 MoAb (eBioscience) for 1 h at 37°C. VSSP (1 μg ganglioside/ml), LPS (0·05 μg/ml), lipohexapeptide Pam_3-Cys-Ser-Lys_4-OH (0·05 μg/ml; Boehringer Mannheim, Mannheim, Germany) or plain culture medium were then added to a final volume of 200 μl. Supernatants were collected after 2 h incubation for tumour necrosis factor (TNF)-α quantification.

Cytokine quantification

IFN-γ, IL-6, IL-10 and TNF-α concentrations in culture supernatant were assessed by enzyme-linked immunosorbent assay (ELISA) in 96-well microtitre plates using MoAbs and recombinant cytokines from Pierce (Rockford, IL, USA) following the manufacturer's instructions. IL-12 p40 and p70 were assessed with OptEIA kits from BD Pharmingen (San Diego, CA, USA).

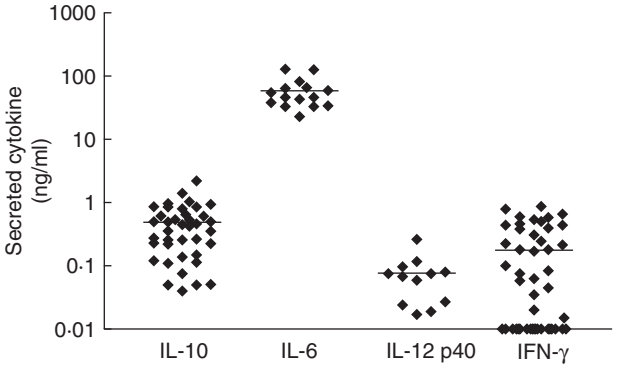

Fig. 1. Cytokine secretion pattern by very small size proteoliposomes (VSSP)-stimulated peripheral blood mononuclear cells (PBMC). PBMC were cultured in the presence of VSSP or medium alone and supernatants were analysed for the presence of interleukin (IL)-10 ($n = 39$), IL-6 ($n = 15$), IL-12 p40 ($n = 12$) and interferon (IFN)-γ ($n = 50$). The scattergram indicates the net cytokine secretion ($cytokine_{VSSP} - cytokine_{medium}$) for each analysed PBMC sample. The mean is indicated by a horizontal bar.

Flow cytometry

DC were harvested with a cell scraper, resuspended in phosphate-buffered saline (PBS) supplemented with 10% FCS and incubated for 30 min at 4°C with fluorescein isothiocyanate (FITC)-labelled anti-CD80 or anti-CD86 antibodies (BD Pharmingen). Cell-bound fluorescence was determined with a fluorescence activated cell sorter (FACS) FACScalibur (Becton Dickinson) flow cytometer.

Statistical methods

Data are expressed in histograms as mean ± standard deviation. Comparison of groups was performed with the Student's unpaired two-tailed *t*-test.

Results

VSSP-induced cytokine secretion

PBMC were cultured in the presence of VSSP (5 μg ganglioside/ml, 6·7 μg protein/ml) or medium alone and supernatants were saved for assessment of cytokine secretion. The supernatant concentrations of IL-10, IL-12, IL-6 and IFN-γ were determined (Fig. 1). A strong IL-6 response was observed (mean: 58 ng/ml). The IL-10 secretion was two logs lower (mean: 488 pg/ml). Mean IL-12 p40 secretion was 77 pg/ml and IL-12 p70 was not detected (sensitivity 4 pg/ml). IFN-γ secretion was detected in 56% of the evaluated PBMC samples (mean: 310 pg/ml in the responder group, $n = 28$). The overall mean for IFN-γ secretion was 177 pg/ml ($n = 50$). Therefore, VSSP induces a mixed pattern of cytokine secretion with a preponderance of IL-6.

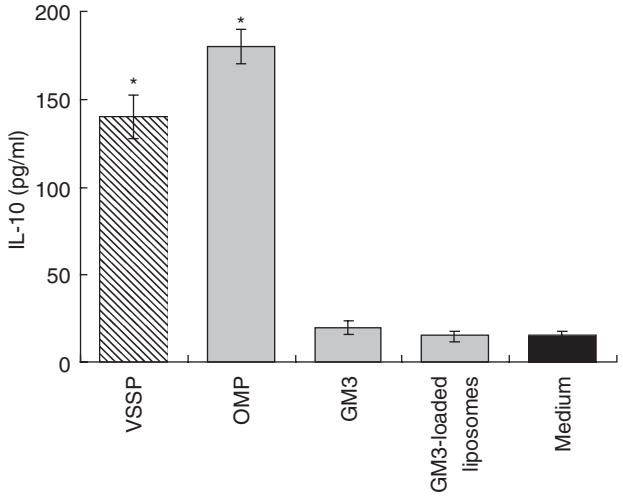

Fig. 2. Interleukin (IL)-10 secretory response to neisserial outer membrane proteins (OMP). Peripheral blood mononuclear cells (PBMC) were cultured in the presence of very small size proteoliposomes (VSSP), 5 μg/ml OMP, 5 μg/ml purified GM3, 5 μg/ml liposome-embedded GM3 or medium alone. Bars represent the mean of triplicate cultures. Standard deviation is indicated in brackets. *$P < 0{\cdot}01$ *versus* medium. Similar results were obtained with PBMC samples from two different donors.

Relative contribution of GM3 and neisserial derivatives to IL-10 secretion

GM3 was reported to induce IL-10 secretion by purified T cells [23]. In order to determine whether the VSSP-induced IL-10 secretion by PBMC was elicited by the ganglioside contained in VSSP or by the neisserial OMP, we also cultured the PBMC in the presence of different GM3 preparations and native OMP. Purified GM3 and GM3-loaded liposomes were unable to induce significant amounts of IL-10 (Fig. 2). Similar results had been obtained by us previously with the same ganglioside preparations after *in vitro* stimulation of PBMC derived from melanoma patients and healthy volunteers (data not shown). In contrast, ganglioside-free neisserial OMP induced at least as much IL-10 as VSSP (Fig. 2). Therefore, although purified T cells were reported to secrete IL-10 after GM3 stimulation, the IL-10 secretion from our VSSP-stimulated PBMC preparations is triggered most probably by the neisserial components.

Involvement of LPS in VSSP-induced cytokine secretion

LPS content in VSSP is 30 μg per mg of protein [24]. We therefore attempted to determine whether the strong PBMC cytokine response elicited by VSSP is induced by LPS. We stimulated PBMC with VSSP or purified *Escherichia coli* LPS in the presence of increasing amounts of the LPS inhibitor polymyxin B. Polymyxin B concentrations that effectively inhibited cytokine secretion induced by 1 μg/ml LPS did not affect the IL-10, IL-6 or IFN-γ (Fig. 3) secretion induced by VSSP. These results suggest that cytokine secretion induced by VSSP is mediated by neisserial components other than LPS.

Cell types involved in VSSP-induced cytokine secretion

Bacterial antigens are strong stimulators of monocytes, macrophages and dendritic cells. We investigated which cell type within the PBMC samples were responsible for secretion of each of the analysed cytokines. PBMC were fractionated by adherence to plastic as described in the Materials and methods section yielding highly purified monocytes (adherent cells) and the bulk of non-adherent cells (comprising mainly T and B lymphocytes and NK cells). We found that the IL-10 (Fig. 4a) and IL-6 (data not shown) secretory responses to VSSP are produced by isolated monocytes. No IL-10 or IL-6 was detected in the supernatant of VSSP-stimulated non-adherent cells.

In contrast to IL-10 and IL-6, IFN-γ was not detected in the supernatants of VSSP-stimulated monocytes. Instead, the IFN-γ secreting activity was recovered in the monocyte-depleted cell fraction. Furthermore, T cell depletion of PBMC samples abrogated the IFN-γ secretory response to VSSP (Fig. 4b). NK cell response to VSSP was examined in purified NK cells and NK cell lines (see Materials and methods). NK cells were responsive to phorbol 12-myristate 13-acetate (PMA)/ionomycin and to poly(I:C) stimulation but not to VSSP stimulation (data not shown). These results suggest that T lymphocytes, rather than NK cells, mediate the IFN-γ response to VSSP.

Involvement of Toll-like receptors in VSSP-mediated monocyte activation

We further pursued the mechanism of VSSP-mediated monocyte activation, investigating which are the involved cell surface receptors. We used blocking monoclonal antibodies to interfere with signal transduction through either TLR4 or TLR2, monitoring whether any of these receptors are involved in VSSP-induced cytokine secretion. PBMC were stimulated with either VSSP or LPS after blocking TLR4. Unlike LPS-induced cytokine secretion, which is abrogated by anti-TLR4 antibodies, VSSP-mediated IL-6 secretion was unaffected (Fig. 5a).

The monocytic cell line THP-1 was used as a model for assessing TLR2 involvement in VSSP-mediated stimulation. Cells were incubated with either VSSP or lipohexapeptide Pam_3-Cys-Ser-Lys_4-OH (known to activate monocytes through TLR2) after blocking TLR2. Both VSSP- and lipohexapeptide-mediated monocyte activation were abrogated after blocking TLR2 (Fig. 5b). However, significant inhibition was not observed after TLR2 blocking when PBMC samples were tested. Several different cell surface receptors are involved in signalling neisserial products [25].

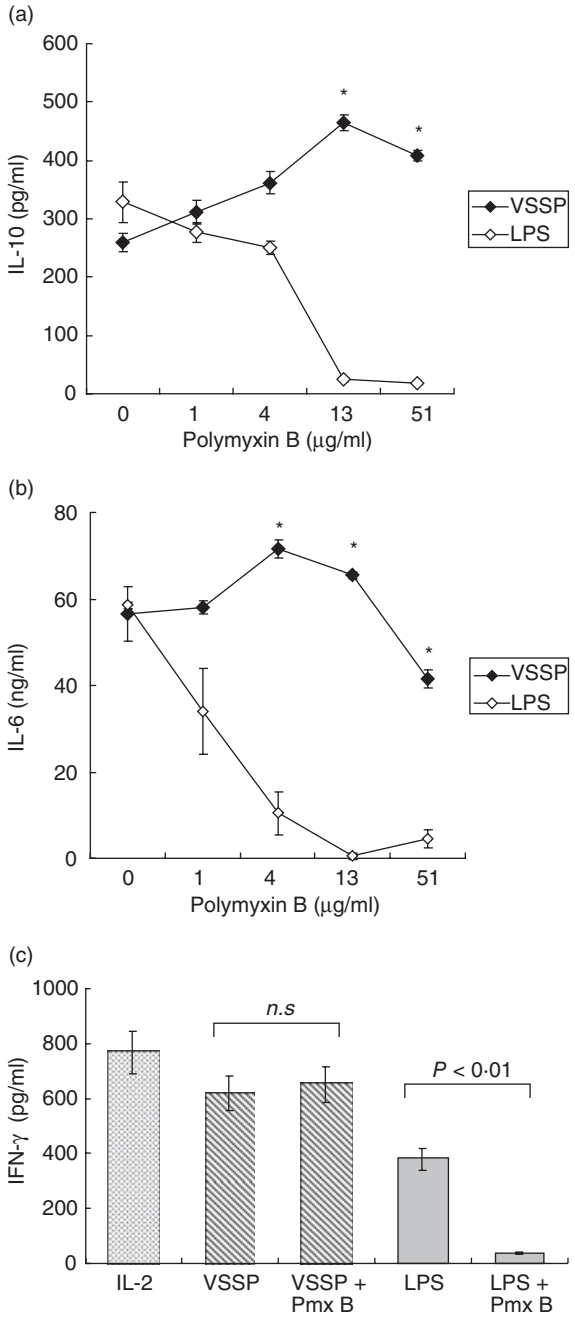

Fig. 3. Very small size proteoliposomes (VSSP)-induced interleukin (IL)-10, IL-6 and interferon (IFN)-γ secretion is not inhibited by polymyxin B (Pmx B). VSSP or lipopolysaccharide (LPS) (0·9 μg/ml) mixed previously with the indicated amounts of Pmx B were added to peripheral blood mononuclear cells (PBMC). Supernatants were collected for quantification of IL-10 (a), IL-6 (b) and IFN-γ (c). IFN-γ secretion assays (c) were performed with 51 μg/ml Pmx B, where indicated. IFN-γ response to addition of 100 U/ml IL-2 is also shown. Results depict the mean net cytokine secretion ($cytokine_{stimuli} - cytokine_{medium}$) from duplicate cultures. Standard deviation is indicated in brackets. *$P < 0{\cdot}01$ *versus* inhibition by Pmx B of LPS-stimulated samples (a,b); n.s.: the difference is not statistically significant. Similar results were obtained with PBMC samples from four different donors.

The stronger TLR2 involvement in VSSP signalling in THP-1 cells might be explained by a differential expression of such receptors. Taken together, these results suggest that VSSP stimulation is mediated mainly by components other than LPS which are ligands to TLR2 and other as yet undefined cell surface receptors.

VSSP-mediated DC maturation

Monocyte-derived immature dendritic cells (iDC) were obtained by *in vitro* culture with IL-4 and GM-CSF-supplemented culture medium. VSSP, LPS or poly(I:C) was added to the culture medium for the last 48 h. iDC spared from any addition were used as a control. Phase contrast microscopy revealed no significant changes in the iDC culture during the last 48 h. In contrast, VSSP addition promoted morphological changes including adhesion, spreading and dendrite elongation (Fig. 6). LPS induced changes similar to those by VSSP. Poly(I:C) provoked the most remarkable cell elongation.

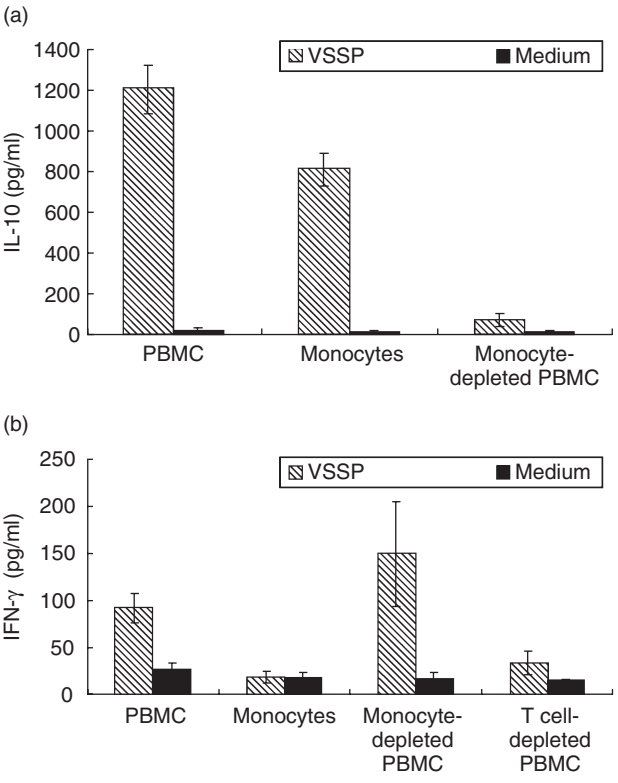

Fig. 4. Very small size proteoliposomes (VSSP) induces monocyte and lymphocyte cytokine secretion. Peripheral blood mononuclear cells (PBMC) were fractionated by adherence to plastic into monocytes, monocyte-depleted PBMC and lymphocyte-depleted PBMC and cultured in the presence of VSSP or medium alone. Supernatants were saved for interleukin (IL)-10 (a) or interferon (IFN)-γ quantification (b). Bars represent the mean of triplicate cultures. Standard deviation is indicated in brackets.

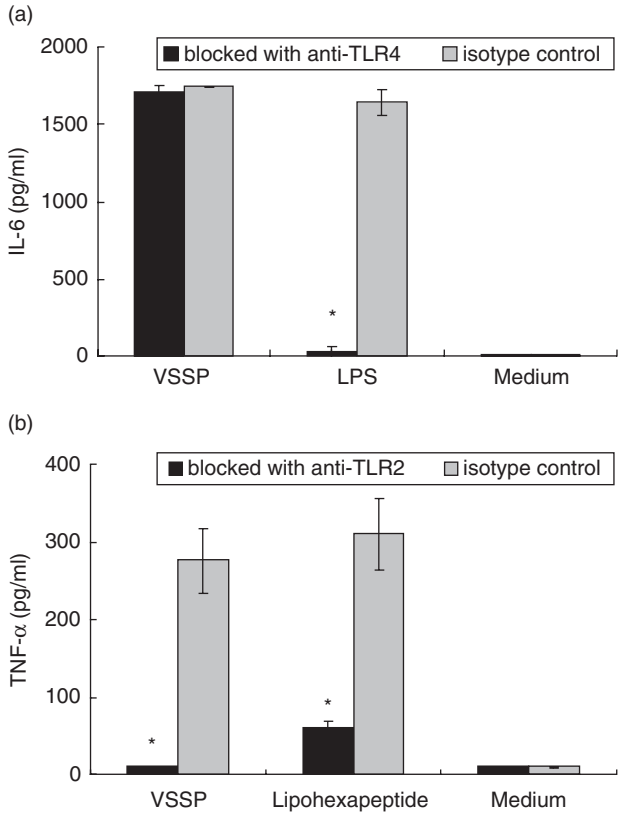

Fig. 5. Toll-like receptor (TLR) involvement in very small size proteoliposomes (VSSP)-mediated monocyte activation. Peripheral blood mononuclear cells (PBMC) were incubated with 20 μg/ml anti-TLR4 monoclonal antibody or isotype control. The cells were then stimulated with either 1 μg/ml VSSP or 0·05 μg/ml lipopolysaccharide (LPS). Supernatants were then collected for interleukin (IL)-6 quantification (a). Differentiated THP-1 cells were incubated with 20 μg/ml anti-TLR2 monoclonal antibody or isotype control. The cells were then stimulated with either 1 μg/ml VSSP or 0·05 μg/ml lipohexapeptide. Supernatants were then collected for tumour necrosis factor (TNF)-α quantification (b). Bars represent the mean of triplicate cultures. Standard deviation is indicated by brackets. *$P < 0{\cdot}01$ *versus* isotype control.

Surface expression of CD80 and CD86 was up-regulated by addition of VSSP to iDC as assessed by flow cytometry (Table 1). Surface expression of these maturation markers was, however, more pronounced when cells were cultured with LPS or poly(I:C) (Table 1).

Cytokine secretion was also assessed in both unstimulated iDC or DC obtained by addition of VSSP, LPS or poly(I:C). Supernatants were screened for IL-10, IL-6, IL-12 p40 and IL-12 p70. The results summarized in Table 1 indicate that poly(I:C) was the strongest promoter of DC maturation and activation as it induced the highest secretion of IL-12 p70. VSSP induced no detectable IL-12 p70 (five independent experiments; ELISA sensitivity 4 pg/ml). VSSP did, however, induce the secretion of IL-12 p40. High levels of IL-6 secretion (at the ng/ml range) were induced by the three stimuli. IL-10 secretion, which was the highest in LPS-stimulated DC, was not induced by VSSP.

VSSP-induced DC maturation was thus observed by cellular morphology and co-stimulatory molecule expression, with a cytokine secretion pattern marked by high levels of IL-6 and lower levels of IL-12 p40.

Discussion

We have shown in this study that VSSP is a strong activator of the human innate immune system. VSSP-stimulated PBMC and monocytes secrete a mixed pattern of cytokines marked by high levels of IL-6 and 100-fold lower levels of IL-10. VSSP also induced PBMC to secrete IL-12 (p40 mean concentration: 100 pg/ml). A number of other adjuvants used in cancer immunotherapy, including CpG-ODN [26,27], imiquimod [28] and OK-432 [10], were also described to induce PBMC IL-12 secretion. Other bacterial derivatives, such as heat-labile enterotoxin [29] and monophosphoryl lipid A [30] (both of which have been used for different immunotherapy applications) have been shown to induce very limited, if any, IL-12 secretion by monocytes or PBMC. Those studies have, however, assessed total IL-12 levels and have not addressed the concentration of the biologically active form of IL-12 (the p70 heterodimer). The p40 homodimer has been described to be a putative antagonist to IL-12 heterodimer in mice. Nevertheless, such activity is unlikely in humans [31,32]. As a result, several authors have used IL-12 p40 secretion as an indicator of proinflammatory responses [33–35]. IL-12 p70 is secreted at a much lower concentration than p40, and often goes undetected in stimulated PBMC [36] and macrophage [37] samples.

With a sensitivity in our ELISA test of 4 pg/ml, we were unable to detect IL-12 p70 in the supernatant of VSSP-stimulated PBMC. Other authors have detected IL-12 p70 in

Table 1. *In vitro* maturation of monocyte-derived dendritic cells.

	Medium (iDC)	VSSP	LPS	Poly (I:C)
Co-stimulatory molecule[a]				
CD80	55	205	572	557
CD86	85	835	1669	1928
Cytokine secretion[b]				
IL-10 (pg/ml)	161	146	2520	833
IL-6 (ng/ml)	0·3	7·0	23	23
IL-12 p40 (pg/ml)	12	888	3220	3060
IL-12 p70 (pg/ml)	< 4·0	< 4·0	39	2080

iDC: immature dendritic cells; IL: interleukin; LPS: lipopolysaccharide; poly(I:C): polyinosinic–polycytdylic acid; VSSP: very small size proteoliposomes. [a]As determined by flow cytometry. Data represent peak fluorescence intensity values (arbitrary units). [b]As determined by enzyme-linked immunosorbent assay testing of culture supernatants. Cytokine concentration expressed in ng/ml for IL-6 and in pg/ml for the other cytokines. Some values for IL-12 p70 secretion are below the detection level (4·0 pg/ml).

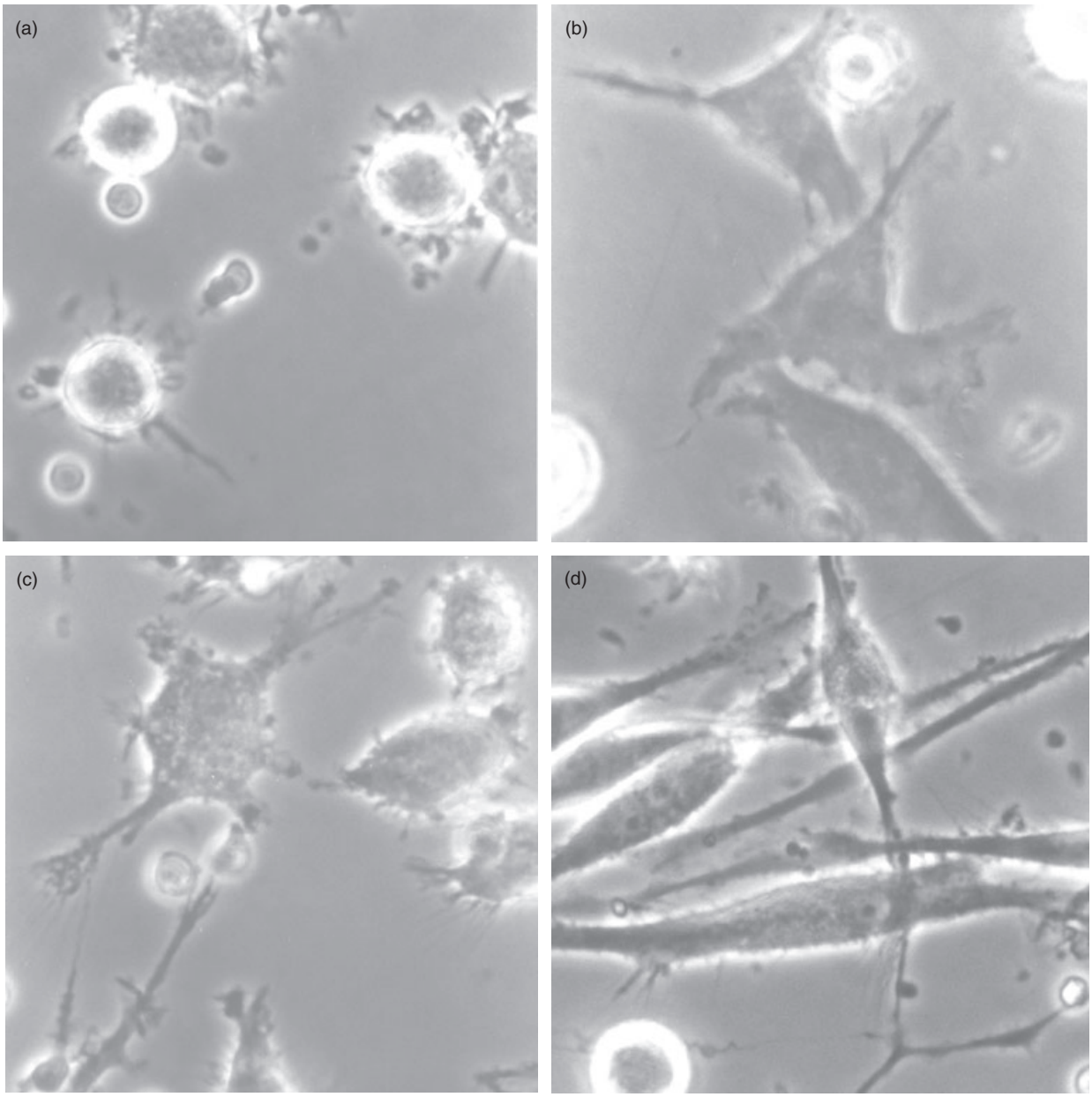

Fig. 6. *In vitro* maturation of monocyte-derived dendritic cells. Adherent monocytes were cultured in the presence of interleukin (IL)-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF) as described in Materials and methods for 8 days to yield immature dendritic cells (a). Alternatively, very small size proteoliposomes (VSSP) (b), lipopolysaccharide (LPS) (c) or polyinosinic–polycytdylic acid [poly(I:C)] (d) were added to the culture medium at day 6.

the supernatant of BCG-stimulated samples with a mean concentration of 2 pg/ml/10^6 PBMC [36]. Such a concentration is beyond our sensitivity limit, and has been recorded with an optimized whole-blood *in vitro* test [36]. Secretion of the Th1-type cytokine IFN-γ by VSSP-stimulated PBMC was attributed to lymphocyte activation and was detected in 56% of the individuals tested. A similar heterogeneity had been noted previously in the response to neisserial cell wall components and attributed to non-LPS components of meningococci that could act as superantigens [38]. The actual active neisserial cell wall components responsible for lymphocyte activation, as well as their signalling pathways, remain to be elucidated and will be the objective of our future work.

Polymyxin B (Pmx B) is a polycation that binds to the negatively charged lipid A moiety of LPS, thereby inhibiting LPS-mediated activation of immune cells. Pmx B is an effective inhibitor of *E. coli* and *Acinetobacter calcoaceticus* LPS. Other types of LPS, such as that derived from Salmonella, have been reported to be unaffected by Pmx B. Inhibition

of *N. meningitidis* LPS by Pmx B is, however, more controversial. Inhibition was observed only at low LPS concentration and in the presence of serum [39]. Pmx B did not affect *N. meningitidis* lipooligosaccharide stimulation of rabbit splenocytes [40]. In contrast, Pmx B inhibited *N. meningitidis* LPS stimulation of mouse splenocytes [41] and human epithelial cells [42]. Stimulation of human whole blood samples showed that Pmx B inhibited 60% of the activity of *N. meningitidis* LPS embedded in outer membrane vesicles [43].

In view of these data, our results showing that of Pmx B was unable to inhibit VSSP-mediated cytokine secretion suggest that components different from LPS may be responsible for cytokine secretion.

Whereas TLR4 is involved in signalling the presence of LPS [44], TLR2 was shown to mediate LPS-independent cellular activation in response to LPS-free Neisseria [45,46], neisserial porins [47], lipoproteins [34] and a variety of other microbial ligands [44]. Neisserial LPS embedded in outer membrane vesicles, however, has been reported to signal through both TLR4 and TLR2 [43]. Because purified LPS signals through TLR4 and not TLR2 [43], it is conceivable that involvement of TLR2 in outer membrane vesicle signalling is triggered by components different from LPS. In line with the latter observations, we have found that TLR2, rather than TLR4, is involved in VSSP-mediated monocytic activation, suggesting that components different from LPS might take part in VSSP-mediated cellular activation. Whether VSSP is capable of signalling through additional cell surface receptors [25] has not been examined.

Extending previous findings [21], we showed that VSSP induces human DC maturation as assessed by cell morphology and co-stimulatory molecule expression. Similar phenotypic differentiation into mature DC were shown to be induced by TNF-α or IL-15 [48]. We have shown in this study that VSSP induced human DC to secrete IL-6 and IL-12 p40. It is noteworthy that in contrast to purified LPS, VSSP did not induce DC to secrete IL-10.

OK-432 was shown to induce human DC to secrete IL-12 p70 [11], as have other OK-432 derivatives such as OK-DNA [49] and OK-PSA [50,51]. However, unlike OK-432, the latter preparations have not been evaluated so far in a clinical setting and their safety profile is still to be determined. On the other hand, assessment of BCG-induced IL-12 p70 secretion by DC gave both positive [52] and negative [53] results. Muramyl dipeptide (a mycobacterium derivative used for treatment of colorectal metastasis) was shown to induce DC to secrete IL-12 p40 levels similar to those induced by VSSP. However, the secretion of p70 was not addressed [54]. Similarly, total IL-12 was measured in polysaccharide K-stimulated DC supernatants [55]. In addition to IL-12 p40, we have analysed DC supernatants for the presence of IL-12 p70. No p70 could be detected in VSSP-stimulated DC supernatants (sensitivity: 4 pg/ml).

It has been shown previously that neisserial proteoliposomes constitute an effective antigen delivery system [56] and that VSSP is indeed an effective adjuvant for different vaccine formulations in mice [20,21,24]. Collectively, our *in vitro* cytokine-secretion and DC-maturation experiments suggest that VSSP is an interesting candidate as an immune stimulant. Further functional studies are required to ascertain its capacity to promote a Th1-type immune response.

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