Molecular BioSystems

Dynamic Article Links

Cite this: *Mol. BioSyst.*, 2012, **8**, 3166–3177

www.rsc.org/molecularbiosystems

PAPER

Activation of monocytic cells by immunostimulatory lipids conjugated to peptide antigens†

Stefania Galdiero,[a] Mariateresa Vitiello,[b] Emiliana Finamore,[b] Rosalba Mansi,[a] Marilena Galdiero,[b] Giancarlo Morelli[a] and Diego Tesauro*[a]

Received 30th September 2011, Accepted 3rd May 2012
DOI: 10.1039/c2mb25064k

Bacterial derived lipoproteins constitute potent macrophage activators *in vivo* and are effective stimuli, enhancing the immune response especially with respect to low or non-immunogenic compounds. In the present study we have prepared branched lipopeptide constructs in which different (B- and T-cell) epitopes of Herpes simplex virus type 1, derived from glycoproteins B (gB) and D (gD), are linked to a synthetic lipid core. The ability of the lipid core peptide (LCP) constructs (LCP-gB and LCP-gD) to induce cytokine expression and activate the mitogen-activated protein kinase cascade has been evaluated and compared with the behaviour of the isolated epitopes and the lipid core. In this respect, the use of LCP technology coupled with the use of three different gB or gD peptide epitopes in the same branched constructs could represent an interesting approach in order to obtain efficient delivery systems in the development of a synthetic multiepitopic vaccine for the prevention of viral infections.

1. Introduction

Subunit protein or peptide vaccines are becoming increasingly popular due to their safety, lack of side effects and easy production.[1,2] However, a major disadvantage with using peptides as vaccines is that they do not generate sufficient immune response. This is because peptide haptens do not trigger the pattern recognition receptors and do not activate the phagocytic cells. Without activation of the enhanced antigen presenting cells (APC), the T and B lymphocytes are not activated and a productive immune response is not initiated.

As a consequence, a critical issue in the development of synthetic peptide vaccines is that, despite containing extremely pure antigens, they tend to be poorly immunogenic compared to live attenuated vaccines.[3,4] Therefore, an important step in the development of epitope-based vaccines is the identification of a safe, efficient, and adjuvant-free antigen delivery system to achieve enhanced antigen presenting cells response.[2]

Conventional vaccine formulations to be administered in animal models require the use of adjuvants such as complete Freund's adjuvant (CFA) or others unsuitable for human use due to their high toxicity,[5] while current vaccines licensed for human use mainly contain alum-based adjuvants.[6] An alternative strategy to overcome the use of toxic adjuvants is to exploit novel self-adjuvating delivery systems. The technology based on lipid core peptide (LCP) constructs represents a potentially safe option for vaccine delivery in humans and gives the possibility to incorporate multiple copies of different glycoprotein peptides.[7]

The LCP system has been described by Moyle and Toth[8] in which lipoamino acids are coupled to a polylysine core containing up to two different peptides of interest at defined positions. It is designed to incorporate the antigen, adjuvant and carrier moieties all in the same molecular entity.[9,10] LCP can be easily synthesized in a single reaction vessel, by step-wise solid-phase methods, without isolating any of the intermediates. The LCP system essentially combines lipidic adjuvant, carrier and multiple peptide epitope. When covalently linked to a peptide, pam_3cys lipopeptide compounds were found to be potent immunogens with self-adjuvating properties, eliciting humoral and cellular responses, irrespective of the route of administration.[11–14] Most importantly, lipopeptide constructs represent potent safe vaccines for human applications.[15] The technology has proven effective in a number of experimental models. In fact, LCP-based vaccine candidates incorporating domains of *Chlamydia trachomatis* outer membrane protein and viral peptides from foot-and-mouth disease virus were immunogenic and resulted in the induction of peptide antibodies in the absence of any additional adjuvant.[16,17] Furthermore, LCP formulation incorporating epitopes from the group A

[a] *Department of Biological Sciences, Division of Biostructures, Centro Interuniversitario di Ricerca sui Peptidi Bioattivi - University of Naples "Federico II", Istituto di Biostrutture e Bioimmagini – CNR, Via Mezzocannone 16, 80134, Naples, Italy. E-mail: diego.tesauro@unina.it; Fax: +39 (0)812534574; Tel: +39 (0)812536643*
[b] *Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, Second University of Naples, 80138, Naples, Italy*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2mb25064k

streptococci (GAS) were tested in murine parental immunization and GAS challenge models. Immunization led to the induction of high-titer GAS peptide-specific serum IgG antibody responses and the induction of high opsonic antibodies. Moreover, mice were protected from GAS infection following challenge.[10,18–22] Another LCP formulation was tested by Haro *et al.* They analysed the immunogenic properties of a tetrameric heterogeneous palmitoyl-derivatised MAP containing two HAV peptide sequences, VP1(11^25) and VP3(102^121), in immunised rabbits.[23]

For the rational design of synthetic vaccines, a potential immunogen must contain appropriate helper T cell and B cell determinants to elicit a strong and relevant immune response. Tam and co-workers[24] have described an innovative method which involves the synthesis of a multiple antigenic peptide in which multiple copies of the same peptide are assembled on a lysine core through the α and ε amino groups. The availability of orthogonally protected lysine derivatives allows the synthesis of peptide containing different determinants with a potential use in the design of multivalent vaccines incorporating different B and T cell determinants. Furthermore, innovative synthetic strategies have been developed by Andreu *et al.* to prepare a multivalent lipopeptide dendrimer incorporating four copies of disulfide-mediated cyclic epitopes which has been successfully used in an experimental vaccination trial against foot-and-mouth disease virus.[25]

Branched synthetic peptide constructs were found to be superior in eliciting antibody when compared to the same determinants assembled in a linear conformation.[26] Moreover, formulation of an effective immunogen that could elicit a potent immune response should incorporate both B cell epitopes that mimic the three-dimensional conformation of the antigen and T cell epitopes resulting from the processed protein, on the same carrier that links the major histocompatibility complex (MHC) with the T cell receptor. Identification of the T and B-cell epitopes is crucial for the development of effective vaccines against infectious agents and different tools are used for their identification. The TEPITOPE prediction software has been shown to be a useful tool in the identification of candidate T cell epitopes in many protein antigen, allowing considerable reduction of the number of peptides to be assayed *in vitro*; moreover, it has been successfully employed to identify HLA-DR ligands derived from tumors,[27,28] endogenous protein involved in autoimmune diseases,[29] viral protein 16 (VP16) of HSV-2[30] and gD of HSV-1.[31] B cell epitopes are located on the surface of proteins usually near γ-turns and were identified using the Chou–Fasman and PHD (Profile network prediction HeiDelberg) prediction methods;[32,33] revealing the hydrophilic sequences with unordered structure or γ-turn.

HSV-1 infections are commonly worldwide between 60% and 90% of the adult population in Europe and USA having HSV-1 antibodies.[34] The prevalence of HSV-2 among adults varies between 20% and 90%, depending upon the country, region within the country and subgroups of population.[34] HSV infections are therefore extremely common and cause a wide range of symptoms, from non-apparent to life-threatening diseases, including genital herpes, orolabial infections (*e.g.* gingivostomatitis, labialis, pharyngitis), cutaneous infections (*e.g.* whitlow, herpes gladiatorum), ocular infections, neonatal herpes, herpes encephalitis, disseminated infection and erythema multiforme.[35] Recurrent genital and orolabial HSV infections cause severe health problems and psychological distress requiring health care. HSV usually initiates infection at the mucosal membrane surface or through small lesions in the skin. During primary infection, the virus replicates at a local site, spreads to and up the weakened nerve, replicates in the dorsal root or trigeminal ganglia and returns to infect the skin along the dermatome. In addition, HSV establishes a latent infection of the neuron, capable of being reactivated by stress, thereby causing recurrent disease.[36,37] Great effort has been devoted to the development of an effective herpes vaccine that could help control this epidemic, through the identification of protective epitopes and development of an effective and safe immunization strategy.

The immune response is the most important defence against HSV infection. Several pieces of evidence, in both animal and human models, suggest that $CD8^+$ T cells play a major role in anti-herpes virus immunity.[38] While $CD8^+$ T cells are useful effectors in the clearance of herpes infections, $CD4^+$ T cells are required to help prime and sustain antiviral $CD8^+$ T-cell immunity.[39] Consequently, an immunogenic formulation should be targeted towards inducing both $CD4^+$ and $CD8^+$ T-cell populations. Possible T and B-cell epitopes were chosen analyzing HSV glycoproteins B and D with several secondary structure prediction methods.

Among the advantages of an epitope based vaccine against a whole protein vaccine is the possibility of including multiple immunodominant and subdominant epitopes and thus eliciting superior responses. In this study, gB and gD, being the two major targets of HSV-1 immune clearance mechanisms, have been selected and analysed to choose candidate antigens.

In this study we have explored the potential of LCP technology to develop a novel self-adjuvating HSV-1 multi-antigen component vaccine delivery system, and tested the hypothesis that different HSV-1 gB and gD epitopes, linked to the lipid core, would stimulate cytokine production and activate the MAPK cascades, pointing to the application of this system in human vaccine development against HSV infection.

2. Materials and methods

2.1. Reagents

All *N*-Fmoc-amino acid derivatives, the HOBt, PyBop, HATU activants, Boc-Gly-PAM resin and 2-chlorotritylchloride resin were purchased from Inbios (Napoli, Italy). All other chemicals were commercially available at Sigma-Aldrich (Milano, Italy) and were used as received unless otherwise stated. RPMI 1640, glutamine, penicillin and streptomycin were purchased from Gibco Life Technologies (Grand Island, NY). Fetal calf serum (FCS) containing <0.01 endotoxin U mL^{-1} was obtained from HyClone Laboratories (Logan, UT).

The FACE kit for the analysis of kinase phosphorylation was purchased from Active Motif (Carlsbad, CA). A cytokine enzyme-linked immunosorbent assay kit was obtained from R...D Systems (Minneapolis, MN, USA). A LAL gel-clot assay was purchased from PBI International (Milan, Italy).

2.2. T and B-cell epitope prediction

The sequences of gB and gD were subjected to HLA-DR ligand prediction by the TEPITOPE software to identify

promiscuous HLA-DR ligands. TEPITOPE is based on 25 virtual matrices that cover a significant part of human class II peptide binding specificity. The TEPITOPE prediction threshold was set at 2% and picked peptide sequences predicted to bind at least four of the seven most common HLA-DR alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1501) were picked. The peptides were chosen based on their predicted binding as well as the degree of similarity between different strains and were synthesized according to the sequence of HSV-1. A peptide present in several strains but showing poor prediction of promiscuous binding was used as a non-promiscuous control.

For B-cell epitope prediction, the sequences of gB and gD were subjected to two different secondary structure prediction methods: the Chou–Fasman algorithm and the PHD method. The Chou–Fasman algorithm predicts the α-helix, β-sheet and other secondary structure based on the occurrence frequency of 20 amino acids in different protein conformations.[40,41] Predictive values were calculated for each type of secondary structure. To calculate these values, the frequency of amino acid j in structure k is divided by the frequency of all the residues in structure k; we obtain primary structural parameters P_α and P_β that range roughly from 0.5 to 1.5 for the 20 amino acids. The algorithm uses the following set of rules to predict protein secondary structure. The sequences are first scanned for small fragments of residues which have a high probability of being the nucleation of a certain type of secondary structure. For α-helices, the core is predicted when four of six residues have a probability >1.03 and $P_\alpha > P_\beta$. For β-strands, three of five residues with the probability >1.00 and $P_\beta > P_\alpha$ predict a nucleation. The nucleations are extended along the sequence in each direction until the average predictive value for four residues drops below 1 for α-helix (1.05 for β-sheets). If both helix and strand are predicted in a certain region (overlapped region), the secondary structure conformation with higher average propensities is predicted. The PHD method uses a neural network algorithm trained and tested on a database of 24 representative protein chains of known structure. Multiple sequence alignments are used as input to a neural network. At the training stage, a database of protein families aligned to proteins of known structure is used. At the prediction stage, the database of sequences is scanned for all homologues of the protein to be predicted, and the family profile of amino acid frequencies at each alignment position is fed into the network. The input signal is propagated through a network with one input, one hidden, and one output layer. The output layer has three units corresponding to the three secondary-structure states, helix, β-strand, and ''loop''.

2.3. Peptide synthesis

All six peptides (two T-cell epitopes and one B epitope for each glycoprotein), fully protected on the amino acid side chains, were synthesized by solid-phase methodology on a 2-chlorotritylchloride resin (1.25 mmol g^{-1}; 1.00 g) using the Fmoc strategy. The resin was swelled in DCM for 30 min and the first amino acid was coupled by adding 1-fold molar excess of Fmoc protected amino acid, mixed with 4-fold molar excess of DIEA in DCM. The suspension was stirred at room temperature for 2 h, then the resin was washed twice with a mixture of DCM/MeOH/DIEA (17/2/1), twice with DCM and finally swelled in DMF. Fmoc was removed using 20% piperidine in DMF. UV-vis measurements of free Fmoc absorption at 301 nm were performed in order to determine the amount of linked amino acid, and therefore the synthesis scale. For all six peptides the synthesis scale was in the 0.45–0.55 mmolar range.

The other amino acids were sequentially coupled by adding 2-fold molar excess of each Fmoc protected amino acid, mixed with equimolar amounts of HOBt, PyBop and 2-fold molar excess of DIEA in DMF. On the amino terminus of $gB_{493–509}$, $gB_{577–599}$, $gD_{119–140}$, $gD_{215–234}$, Boc protected glycine was added in order to obtain Boc protected peptides. The coupling reaction proceeded at room temperature for 2 h under stirring, and was monitored with the Kaiser ninhydrin test. Unreacted amino groups were capped using a mixture of DMF/acetic anhydride/pyridine (91.3/4.7/4). The fully protected peptides were cleaved from the solid support by suspending the resin in a mixture of TFA/(TIS)/DCM (1/5/94) for 2 min; then the solution (10.0 mL) was filtered into a flask containing 10% pyridine in MeOH (2 mL). This cleavage procedure was repeated up to 10 times, the filtrates were combined and evaporated under reduced pressure to 5% of the initial volume. The crude products were precipitated by adding cold water, and purified by RP-HPLC on a Shimadzu 8A apparatus equipped with an UV Shimadzu detector using a Phenomenex C18 column (Torrance, CA, USA) 22 × 250 mm, eluted with the H_2O–0.1% TFA (A) and CH_3CN–0.1% TFA (B) mixture; a two step gradient was used: from 60% to 80% of B in 10 min and from 80% to 95% of B in 15 min with a flow rate of 20 mL min^{-1}. The purified peptides were characterized by LC-ESI-MS using a Finnigan Surveyor MSQ single quadrupole electrospray ionisation mass spectrometer coupled with a Finnigan Surveyor HPLC (Finnigan/Thermo Electron Corporation San Jose, CA, USA) or by MALDI-TOF on a MALDI-TOF Voyager-DE Perseptive Biosystem (Framingham, MA). The six peptides fully deprotected on the amino acid side chains were also obtained by cleaving from the resin with TFA/TIS/H_2O (95.5/2.5/2) mixture and HPLC purification. The molecular masses for each peptide, in the protected or deprotected form, are reported in Table 1.

2.4. Synthesis of LCP constructs

Synthesis of the two LPC systems was carried out using solid-phase methodology with a combination of Fmoc and Boc based strategies. Pre-loaded Boc-Gly-PAM resin (1 mmol) was used. The first five residues starting from the C-terminus (two residues of Boc–C_{12}–OH (Boc–$NH_2CH[(CH_2)_9CH_3]COOH$) one residue of Boc-Gly-OH, one residue of Boc–C_{12}–OH and one residue of Fmoc-Lys(Mtt)-OH) were sequentially coupled using the following coupling procedure: single coupling, with the addition of 2 equivalents of protected amino acids activated with 2 equivalents of HATU and 4 equivalents of DIEA in the DCM–DMF mixture, and stirring for 30 min. Boc deprotection steps were performed with 33% TFA/DCM (2 + 20 min). To obtain the branched construct, Fmoc and Mtt protecting groups from the lysine residue were selectively removed,

Table 1 Amino acid sequences of the T-and B-cell epitopes and characterization data of the six synthesized peptides. Found molecular weights are reported for fully protected (f.p.) and fully deprotected (f.d.) peptides. HPLC retention times are reported for the six fully deprotected peptides

Protein fragment	Amino acid sequences	Epitope	M_w (f.d. peptides)	M_w (f.p. peptides)	Rt (min) (f.d. peptides)	Yield (%)
$gB_{308–316}$	HTEHTSYAA	B	m/z 1018	m/z 1527	8.59	55
[Gly]$gB_{577–599}$	GAADNVIVQNSMRISSRPGACYSR	T	$m/2z$ 1277 $m/3z$ 851	m/z 3765	12.03	56
[Gly]$gB_{493–509}$	GERIKTTSSIEFARLQFT	T	$m/2z$ 1042 $m/3z$ 695	m/z 3096	12.39	60
$gD_{301–309}$	SALLEDPVG	B	m/z 899	m/z 1293	9.53	60
[Gly]$gD_{119–140}$	GNLTIAWFRMGGNCAIPITVMEY	T	m/z 2556	m/z 3245	15.10	54
[Gly]$gD_{215–234}$	GKYALPLRIPPSACLSPQAYQ	T	m/z 2271	m/z 2957	21.50	57

and Fmoc-Lys(Dde)-OH and Fmoc-Lys(Mtt)-OH sequentially coupled on the alpha and epsilon free amino functions. In particular, the Fmoc protecting group was removed using a solution of 20% piperidine in DMF and Fmoc-Lys(Dde)-OH was coupled to the alpha amino function. After removal of the Mtt-protecting group, using a mixture of TFA/TIS/DCM (1/5/94), the Fmoc-Lys(Mtt)-OH was assembled as described above. After removal of Fmoc and the Dde amino protecting group, a small quantity of dried Lys_3-LCP-resin was treated with TFA (1 mL) and TIS (3%) and the mixture stirred at room temperature for 5 min. The mixture was then cooled in an ice bath and TFMSA (100 μL) added dropwise with stirring. The flask was sealed with a stopper and the mixture stirred at room temperature for 2 h. The volume was reduced under vacuum and the mixture was cooled in an ice slush bath. The crude product was precipitated adding diethyl ether. The liquid phase was removed by filtration, and the solid dissolved in 50% (v/v) water–acetonitrile. The crude product was recollected after lyophilization and purified by RP-HPLC (from 5% to 70% of B in 30 min with a flow rate of 20 mL min^{-1}) on a semipreparative C_{18} column and characterized by MALDI-TOF (M_w = 1101).

The branched construct with three different peptides was obtained using the three orthogonal amino protection groups: Fmoc, Mtt and Dde. The Fmoc-deprotection strategy involved treatment of the protected peptide-resin with 2% DBU/2% piperidine/NMP (5 min), followed by another 5 min treatment with 20% (v/v) piperidine in NMP; the Mtt protecting group was removed using a mixture of TFA/TIS/DCM (1/5/94); while the Dde protecting group was removed using 0.90 mmol of $NH_2OH{\cdot}HCl$ and 0.675 mmol of imidazole in 2.5 mL of NMP. In detail, 60 mg of Lys_3-LCP-resin (30 μmol) were suspended in DMF and the Dde protecting group was removed as above. Two equivalents of the first fully protected peptide (60 μmol), with a Boc protecting group on the N-terminus, was coupled using the same excess of HATU and 4 equivalents of DIEA. After removal of Mtt-protecting groups, the second peptide (60 μmol) was coupled, as described above, with the appropriate protecting groups on the side chains of the amino acids and the Boc-protecting group on the N-terminus. The third peptide (120 μmol) was coupled on the two α amino groups initially protected with the Fmoc group. Following synthesis, the constructs were removed from the resin by TFMSA cleavage as previously described. The crude constructs were purified by HPLC (from 5% to 70% of B in 30 min with a flow rate of 20 mL min^{-1}) on reverse phase (yields LCP-gB 30%, LCP-gD 32%). ESI mass-spectroscopy confirmed the product identity:

M_w(LCP-gB) = 7698.6 amu; $[M + 5H^+]/5$ = 1540 amu; $[M + 6H^+ + Na^+]/7$ = 1103.8 amu; $[M + 8H^+]/8$ = 963.4 amu; $[M + 9H^+]/9$ = 857.1 amu. (MALDI TOF 7699 amu). HPLC t_r 19.18.

M_w(LCP-gD) = 7654 amu; $[M + 6H^+]/6$ = 1277.1 amu; $[M + 8H^+]/8$ = 957.9 amu; $[M + 9H^+]/9$ = 851.6 amu. (MALDI TOF 7654 amu). HPLC t_r 21.88.

2.5. Cell lines

U937 monocytes (ATCC CRL-1593.2) were grown at 37 °C in 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated FCS, glutamine (2 mM), penicillin (100 U mL^{-1}), and streptomycin (100 U mL^{-1}) and differentiated as previously described.[42] RAW 264.7 cells, a murine macrophage line, were obtained from ATCC (TIB-71™). Cells were cultivated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin. The cells were maintained at 37 °C with 5% CO_2 in a humidified incubator.[43]

Before treatment of the cells, the serum concentration was reduced to 5% for 24 h at 37 °C and then further reduced to serum free media for at least 10–12 h. This should almost eliminate interference from serum factors in the phosphorylation state of the proteins in the signalling cascade.

2.6. Analysis of kinase phosphorylation

U937 differentiated cells and RAW 264.7 cells (1.2×10^5 cells per well) were grown in 96-well plates in accordance with the manufacture's protocol so that they will be approximately 80% confluent at the time of stimulation. Cells were stimulated to induce the pathway of interest at 37 °C with 10 μM of each single peptide, 2.5 μM of each LCP construct and 2.5 μM of Lys_3-LCP, LCP alone, and Lys_3-LCP with non-covalently attached gB and gD peptides (LCP/gB and LCP/gD) for 10 min. The optimal time of stimulation and amount of stimuli used in all assays were determined in preliminary experiments (data not shown). Following stimulation, the cells were rapidly fixed to preserve activation-specific protein modifications. Each well was then incubated with a primary antibody that recognized either phosphorylated or total p38, JNK and ERK 1/2. Subsequent incubation with secondary HRP-conjugated antibody and developing solution has provided a quantified colorimetric readout. Phosphorylated p38, JNK and ERK1/2 detection was performed according to the manufacture's instructions.

2.7. Treatment of U937 and RAW 264.7 cells with specific MAPK inhibitors

In some experiments, before exposure to stimuli, U937 and RAW 264.7 cells were pretreated with different inhibitors: 4-(4-fluorophenyl)-5-(4-pyridyl) 1*H*-imidazole (SB203580; Calbiochem-Novabiochem GmbH, Schwalbach, Germany) (10 μM for 1 h), a specific inhibitor of the p38 pathway;[44] 2′-amino-3′-methoxyflavone (PD-098059; New England Biolabs, Inc.) (100 μM for 1 h) a highly selective inhibitor that blocks ERK1/2 activation by specifically inhibiting MEK1, a kinase that catalyses ERK1/2 phosphorylation;[45] JNKinhibitor-1 (L-JNKI1; Alexis Biochemicals, San Diego, CA) (1 μM for 1 h), a peptide that specifically binds to and inhibits JNK activity.[46] The inhibitors were prepared in dimethyl sulfoxide at a final concentration of 0.1% (v/v).

2.8. Enzyme-linked immunosorbent assay for cytokine

Culture supernatants for ELISA were collected after 24 h following incubation of U937 and RAW 264.7 cells (3×10^6 cells per mL) with stimuli. The optimal concentrations of stimuli (10 μM of each peptide, 2.5 μM of each construct and 2.5 μM of the following controls: Lys_3-LCP, LCP, LCP/gB and LCP/gD) and time point of stimulation have been selected using preliminary experiments (data not shown). After incubation, the samples were centrifuged at 1800 rpm at 4 °C for 10 min, and the supernatants were collected and stored at −70 °C. IL-6, IL-8 or the functional homolog of human IL-8, mouse macrophage inflammatory peptide-2 (MIP-2), and TNF-α were analyzed. Cytokine release was measured according to the manufacturer's recommendations. The assay employs an antibody specific for each cytokine coated on a 96-well plate. Standards, samples and biotinylated anti IL-6, IL-8 or MIP-2 and TNF-α were pipetted into the wells and cytokine present in the samples was captured by the antibody immobilized to the wells and by the biotinylated specific detection antibody. Standard and sample dilutions were added in duplicate wells to each plate. All analyses were performed at least three times for each individual cell-stimulation assay.

2.9. Endotoxin contamination

All solutions and peptide preparations used in our experiments were tested for the presence of endotoxin using a LAL assay as described by Yin and others.[47] The lower detection limit of this assay was 0.1 EU mL^{-1}.

2.10. LDH assay

LDH assay was carried out according to the manufacturer's instructions using a cytotoxicity detection kit (Roche Diagnostic SpA, Milano, Italy). LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into cell culture supernatant when the plasma membrane is damaged. LDH activity was determined by a coupled enzymatic reaction whereby the INT was reduced to formazan. An increase in the number of dead or damaged cells resulted in an increase in LDH activity in the culture supernatant. The amount of LDH shows that treated and untreated cells are healthy.

2.11. Statistical analysis

Each experiment was performed in triplicate. The data are expressed as the mean ± SEM and were analyzed using Student's *t* test. Results were considered to be statistically significant at *P* values of ≤ 0.01.

3. Results

3.1. Identification of potential T- and B-cell epitopes (of HSV-1 gB and gD)

In order to choose the possible T- and B-cell epitopes to conjugate to the lipid core, HSV-1 gB and gD have been analysed with several prediction programs and the data obtained have been compared. The identification of T-cell epitopes is crucial for the development of effective vaccines. As T-cell epitopes only encompass a very small region of a complex protein, the mapping of the dominant T-cell epitopes of an antigen is a difficult procedure. To identify the possible T-cell epitopes, in this study the TEPITOPE prediction program has been used.

The goal of T-cell epitope prediction is to accurately identify peptide sequences within any protein that, in the context of a defined MHC molecule, will elicit desired T-cell responses. For peptide vaccination, these epitopes should ideally be dominant and promiscuous, so that they are recognised by most individuals within an outbreed population. We investigated whether promiscuous HLA-DL ligands could be identified in the sequences of the HSV-1 glycoproteins gB and gD. We used a stringent threshold setting, which was proven to be effective in predicting up to 80% of an *in vitro*-selected peptide repertoire. The selected T-cell epitopes for each glycoprotein are reported in Table 1. We selected two peptides for each glycoprotein with a strong prediction of promiscuous binding to HLA-DR. To the nonamer selected by the T epitope as the core HLA-binding motif, two amino acids were added at the terminal moieties to increase the efficacy of the *in vitro* peptide presentation to the $CD4^+$ T cells. When there was the superimposition of more than one hypothetical HLA-binding frame, longer sequences were synthesized. Moreover, the addition of polar amino acids at the C or N terminus was performed in order to have soluble peptides.

In particular, with our stringent threshold, we selected two peptides for gD (*i.e.* gD119–140 and gD215–234); both belonging to the external N-terminal portion of gD and mapped to nonglycosylated regions of gD. From the analysis of glycoprotein gB with the same threshold we obtained two overlapping epitopes which correspond to the longer peptide gB577–599 and the other epitope gB493–509. The peptide gB577–599 is located in Domain IV while gB493–509 is located in Domain II both peptides being located on the protein surface.

B-cell epitopes are located on the surface of proteins, usually near β-turns. To identify the possible B-cell epitopes several parameters were compared such as hydrophobicity, solvent accessibility and the secondary structure and hydrophilic sequences with unordered structure or β-turn were chosen. Glycoproteins gB and gD have been analyzed by Chou–Fasman and PHD (Profile network prediction HeiDelberg) prediction methods. These secondary structure prediction methods are

based on the propensities of amino acid residues to form β-strands, helices or turns.

On analyzing our results we decided to select one B epitope for each glycoprotein and from those obtained we selected gB308–316 and gD301–309. The peptide gB308–316 is located in Domain I; the peptide gD301–309 is located closest on the hydrophobic membrane anchor domain of gD. Conformational analysis of the gD301–309 (SALLEDPVG) sequence confirms the presence of a type I β-turn structure which includes the DPVG epitope core.[48] Moreover, previous studies indicated that the gD301–309 epitope induced gD-specific responses and also elicited an immune response that conferred protection against lethal HSV-1 infection.[49]

3.2. LCP-gD and LCP-gB constructs

The chemical structures of the two LCP constructs, LCP-gD and LCP-gB, are reported in Fig. 1, and the amino acid sequences of the six peptides corresponding to selected T- and B-cell epitopes for HSV-1 gB and HSV-2 gD are indicated in Table 1.

The two LCP constructs were synthesized, as shown in Fig. 2, using solid-phase techniques based on a combination of Fmoc and Boc strategies, orthogonal protection of the lysine residues, and fully protected peptides, previously synthesized by solid-phase peptide synthesis. This divergent strategy enabled us to obtain a homogeneous product. In fact all epitopes were preventively purified by HPLC before the lipid core coupling.

Synthesis of the lipid core system (Boc-C12-C12-Gly-C12-Gly-PAM resin) was performed manually in solid-phase under standard conditions by using a Boc strategy. Pre-loaded Boc-Gly-PAM resin was used. This resin is usually the support of choice for the preparation of branched peptides by Boc based solid phase methodologies; it is more stable under acid conditions, during the Boc removing cycles, than the corresponding Merrifield resin. The α-aminododecanoic acid H_2N–CH[$(CH_2)_9CH_3$]–COOH (C12) was obtained as described in the literature[50] and protected on the N-terminal function with the Boc group, in order to be used in solid phase synthesis.

The three-lysine branched system was built on the lypophilic moiety, capable of bearing four peptide molecules, as schematized in Fig. 2. The construct was designed to obtain molecules bearing three different epitopes, therefore two of the four peptide sequences, in each construct, are identical. This result was achieved using three orthogonal protecting groups on the four amine functions present on the lysine branched system. Therefore, the synthetic approach initially required, the binding of Fmoc-Lys(Mtt)-OH, and, secondary, the binding of Fmoc-Lys(Mtt)-OH and Fmoc-Lys(Dde)-OH, after the sequential

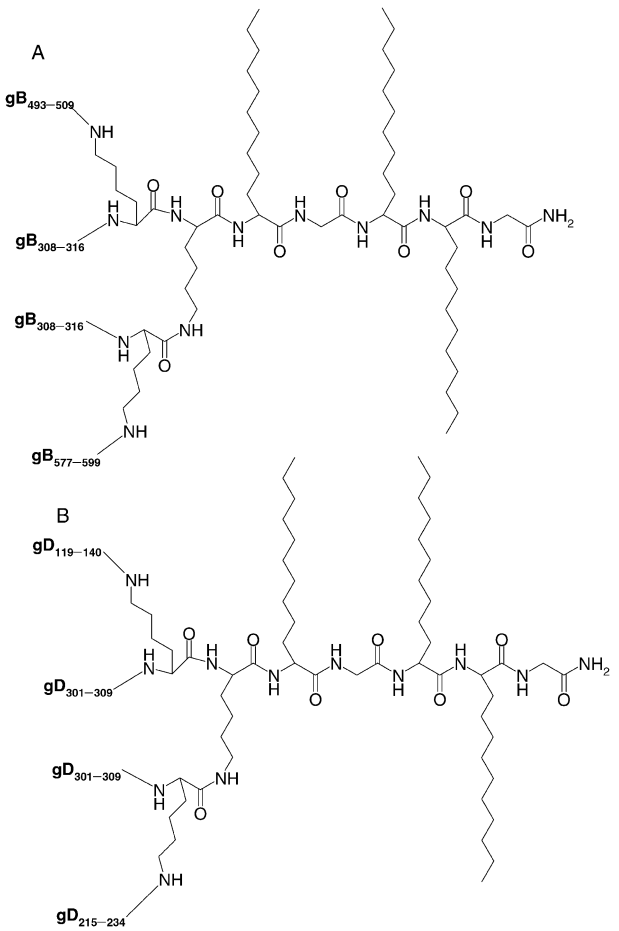

Fig. 1 Chemical structure of the lipid core peptide (LCP) constructs. Panel A shows the structure of LCP conjugated with gB peptides (LCP-gB); panel B shows the structure of LCP conjugated with gD peptides (LCP-gD). Peptide sequences are reported in Table 1.

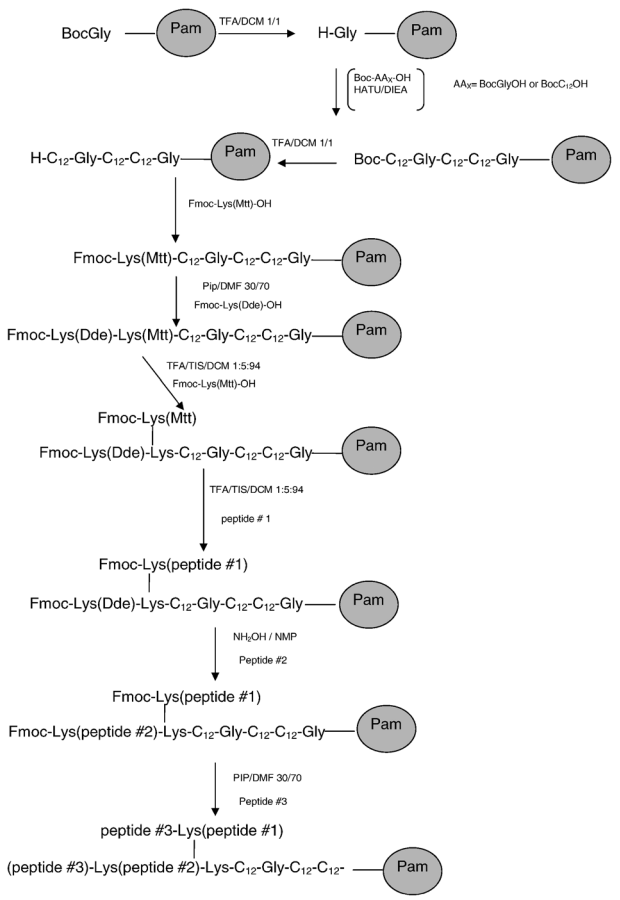

Fig. 2 Synthesis scheme for the preparation of LCP-gB and LCP-gD constructs. The branched peptide conjugates were synthesized using a combination of Fmoc and Boc solid-phase techniques, orthogonally protected lysine residues, and fully-protected peptides.

removal of Fmoc and Mtt protecting groups, respectively. Sequential cleavage of the orthogonal protecting groups allowed the sequential coupling of the fully protected peptides chosen to build up the branched peptide constructs.

The peptide epitopes were synthesized on 2-chlorotritylchloride resin by Fmoc chemistry and purified in solution by HPLC methods. On the amino terminus of $gB_{493–509}$, $gB_{577–599}$, $gD_{119–140}$, $gD_{215–234}$, a Boc protected glycine was added in order to obtain Boc protected peptides. The superacid-labile resin was used to obtain fully protected peptides at the peptide-resin cleavage step. Protected peptides were coupled on the lysine branched lipid core bound to the resin, without any undesired side reaction. The LCP constructs were removed from the resin by TFMSA cleavage. The cleavage from the PAM resin by TFMSA enabled the recovery of the LCP constructs fully deprotected on the peptide side chains.

The two LCP constructs were purified by HPLC and their purity and identity were confirmed by analytical RP-HPLC (figures reported in ESI†), ESI-MS spectrometry (Fig. 3A and B) and MALDI TOF spectrometry (figures reported in ESI†). The final product yields were in the range of 30–40%.

Endotoxin levels of cell cultures and peptide solutions resulted in, LAL tests, lower than 0.1 EU mL^{-1} (data not shown).

The six single isolated peptides, the lipid core containing only the three lysine branched sequence with the lysine amino functions still protected (Lys_3-LCP) and the non-covalent mixtures containing Lys_3-LCP and gB or gD peptides were also prepared to be used in comparative experiments.

3.3. MAPK pathway activation

Among the most activated signal transduction pathways involved in the immune response are the mitogen-activated protein kinase pathways. MAPKs are a group of serine/threonine protein kinases which can be activated by various extracellular stimuli. MAPKs play a key role in the regulation of gene expression and cytoplasmic activities. Three major MAPK modules exist in mammalian species: JNK, ERK and the p38 mitogen-activated protein kinase (p38). The activation of the terminal kinase results in nuclear translocation and presents binding of transcription factors resulting in the gene expression of mediators involved in the inflammatory and immunological response.[51]

We tested the ability of the two constructs and of isolated corresponding peptides to activate p38, JNK and ERK1/2 pathways in U937 and RAW 264.7 cells. Results show that the lipid core alone (LCP), the lysine-lipid core (Lys_3-LCP), the two constructs containing the HSV peptides conjugated to the lipid core (LCP-gB and LCP-gD) and the lysine-lipid core with non-covalently bound peptides (LCP/gB and LCP/gD) were able to activate all the pathways assessed, although with different intensities. Among the kinases studied, ERK1/2 was activated at higher levels by the three constructs used. Induction was about 4–5 fold higher compared to the single isolated peptides (Fig. 4 and 5).

3.4. Cytokines release in LCP-stimulated U937 and RAW 264.7 cells

It has been well demonstrated that lipoproteins or synthetic lipopeptides, such as mycoplasma fermentans-derived macrophage-activating lipopeptide 2, a toll-like receptor 2 agonist, stimulate both human and murine macrophages and induce the secretion of proinflammatory cytokines *via* the activation of MAPK pathways.[52] We have therefore tested the release of IL-6, IL-8 or MIP-2 and TNF-α by immune-responsive cells in the form of isolated U937 and RAW 264.7 cells. The supernatant of U937 and RAW 264.7 cell cultures was tested by ELISA for the presence of secreted IL-6, IL-8 or MIP-2 and TNF in response to different lipopeptide constructs and compared with that induced by the Lys_3-LCP system alone. We also compared the release of cytokines after stimulation with peptides with the release obtained when stimulating cells with the lipidic constructs containing the same peptides non-covalently attached to the lysine-lipid core. Optimal concentrations (10 μM of each peptide; 2.5 μM of each construct; 2.5 μM of LCP; 2.5 μM of LCP/gB and LCP/gD) and time point of stimulation have been selected by preliminary experiments. For stimulation experiments, RAW 264.7 murine macrophage cell line and U937 human monocytic cell line were seeded at 3×10^6 cells per mL density and then treated for a 24 h period. The various treatments performed were not toxic for the cells; in fact, they did not induce any significant release of LDH in the cell supernatants (data not shown).

As shown in Fig. 6, the IL-6, IL-8 or MIP-2 and TNF-α release increased considerably when cells were treated with LCP-gB or LCP-gD constructs compared to the production of the same cytokines when the stimulus was represented by the single or mixed peptides. U937 cells were more reactive to such stimulation, and reached higher levels of cytokine production compared to RAW 264.7 cells. We could not observe any considerable difference between the constructs bearing gD or gB derived epitopes. A minor increase of cytokine production was observed in comparison with the Lys_3-LCP construct alone and with LCP/gB and LCP/gD.

3.5. Effect of MAPK inhibitors on cytokine release in U937 and RAW 264.7 cells

The production of IL-6, IL-8 or MIP-2 and TNF-α is at least in part dependent on MAPK activation. To address whether the treatment of cells with MAPK pathways inhibitor disrupts the axis MAPKs/gene expression of IL-6, IL-8 or MIP-2, TNF-α in U937 and RAW 264.7 cells treated with LCP-gB/gD constructs, we have blocked ERK1/2, JNK and p38 activation using specific inhibitors for 1 h before treatment constructs. With the cytokines produced were modestly but significantly decreased by the presence of specific inhibitors (Fig. 7).

The effect of the ERK1/2 inhibitor PD-098059 was in percentage the same for the release of all cytokines analysed and higher than the effect obtained using p38 and JNK specific inhibitors in both cell lines tested. JNK specific inhibitor reduced cytokine release by 20–25%, while the p38 inhibitor reduced cytokine release to a lower level. These data suggest that both U937 and RAW 264.7 cells produce IL-6, IL-8 or MIP-2 and TNF-α at least partially through the activation of ERK1/2, JNK and p38 in response to the synthesized constructs.

4. Discussion

Great effort is devoted to the development of an effective herpes vaccine that could help control this epidemic, through the identification of protective epitopes and development of an

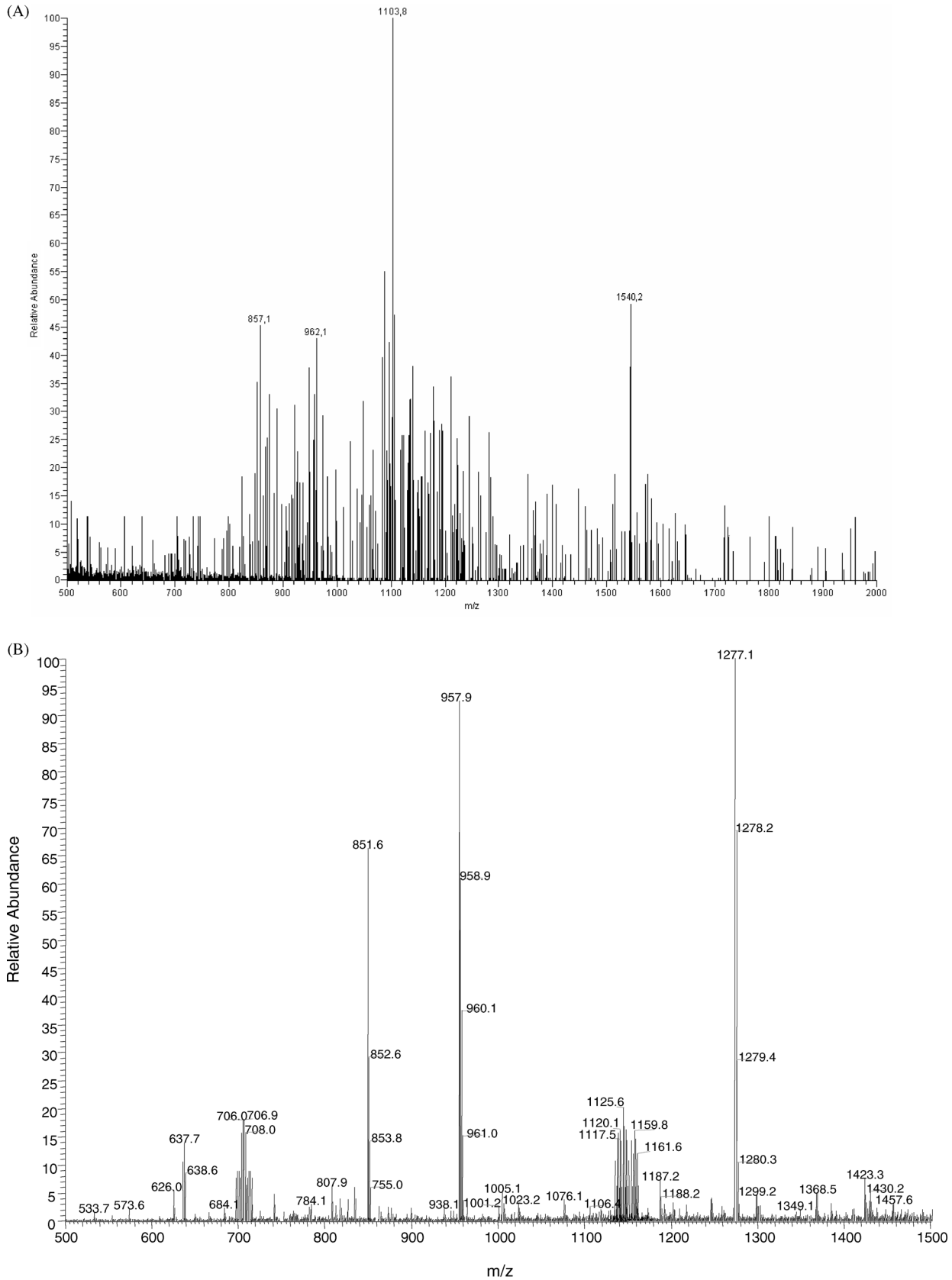

Fig. 3 ESI mass spectra of: (A) LCP-gB and (B) LCP-gD constructs.

effective and safe immunization strategy. The focus of this study was to explore the capacity of new LCP constructs containing multiple copies of different peptides, derived from HSV-1 glycoproteins f, gB or gD epitopes, to enhance the immune

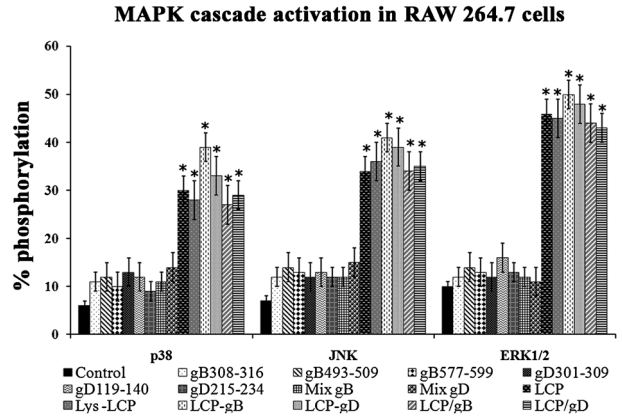

Fig. 4 MAPK cascade activation in RAW 264.7 cells in response to a dose of 10 μM of each single isolated peptide; 2.5 μM of each LCP construct; 2.5 μM of LCP; 2.5 μM of Lys_3-LCP; 2.5 μM of LCP/gB and LCP/gD and at a stimulation time of 10 min. Activation of each MAPK subfamily was determined by a quantitative assessment of enzyme activity. The data are represented as mean ± standard error. * $P \leq 0.01$ indicates statistically significant difference *versus* untreated cells or single/mixed peptides pretreated cells (Student's *t*-test).

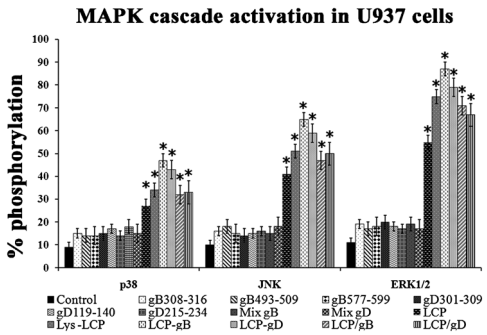

Fig. 5 MAPK cascade activation in U937 cells in response to a dose of 10 μM of each peptide; 2.5 μM of each LCP construct; 2.5 μM of LCP; 2.5 μM of Lys_3-LCP 2.5 μM of LCP/gB and LCP/gD and at a stimulation time of 10 min. Activation of each MAPK subfamily was determined by a quantitative assessment of enzyme activity. The data are represented as mean ± standard error. * $P \leq 0.01$ indicates statistically significant difference *versus* untreated cells or single/mixed peptides pretreated cells (Student's *t*-test).

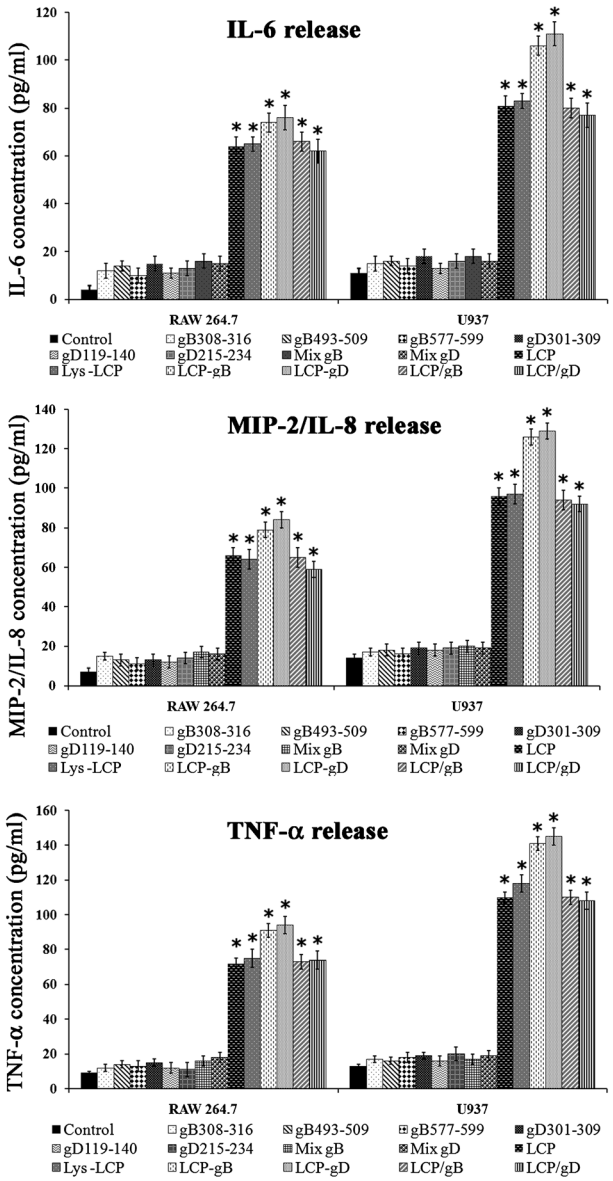

Fig. 6 IL-6, IL-8 or MIP-2 and TNF-α release after treatment with 10 μM of each peptide, 2.5 μM of each LCP construct; 2.5 μM of LCP; 2.5 μM of Lys_3-LCP; 2.5 μM of LCP/gB and LCP/gD for 24 h. The results shown are the average of three independent experiments, and the error bars indicate the standard errors of the means. * $P \leq 0.01$ indicates statistically significant difference *versus* untreated cells or single/mixed peptides pretreated cells (Student's *t*-test).

response evaluating the cytokine production by macrophages mediated by MAPK cascades.

Of the known HSV glycoproteins, gB and gD represent excellent candidates for inducing a protective immunity in animal models. The palmitoyl-tailed HSV-gB498-505–Pan HLA-DR-binding epitope, a chimeric epitope delivered in adjuvant free saline, induced potent virus-specific IFN-γ-producing $CD8^+$ cytotoxic T lymphocytes in H2b mice.[53] Nesburn *et al.*[49,54] have presented a vaccine strategy that consists of ocular mucosal delivery of peptide epitopes, selected from the glycoprotein gD of HSV-1 mixed with synthetic immunostimulatory oligodeoxynucleotides containing unmethylated CpG motifs, capable of inducing peptide-specific and virus-neutralizing IgA/IgG in tears as well as in serum. These studies as well as the recently identified T-cell epitopes in gD and gB that induce potent HSV-specific IFN-γ-producing $CD4^+$ T cells,[7,55] prompted us to evaluate the T-cell-mediated protective efficacy of gB and gD epitopes linked to a lipid core. Experimental data demonstrate that LCP technology has the ability to develop constructs capable of altering cell response and may be of particular advantage for vaccine design in pathogenic immune response by HSV-1.

A multi-epitope-based herpes vaccine could thus include several T-cell epitopes present in several different structural glycoproteins and regulatory proteins chosen to represent the HLA supertypes known to provide recognition in most individuals within an outbred population.

In this study, in order to choose potential T-cell and B-cell epitopes, gB and gD of HSV-1 have been analysed with several prediction programs and the data obtained have been compared.

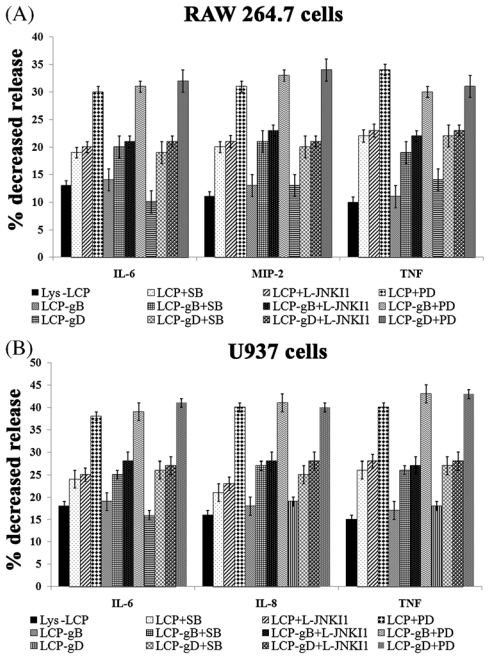

Fig. 7 Analysis of the effects of the SB203580 (SB; p38 inhibitor), PD-098059 (PD; ERK1/2 inhibitor) or L-JNKI1 (JNK inhibitor) on IL-6, IL-8 or MIP-2 and TNF-α release induced in U937 cells (A) and RAW 264.7 cells (B). The results shown are the average of three independent experiments, and the error bars indicate the standard errors of the means.

Our experimental data demonstrate that LCP peptide technology, associated with TEPITOPE prediction, has the ability to alter the quality and quantity of an immune response and may be of particular advantage for vaccine design in pathogenic immune response by HSV-1. In recent years, a wide variety of proteins have been used as carriers for the preparation of synthetic peptide-based vaccines. These types of vaccines have advantages, over traditional approaches, being selective, chemically defined and safe. Carriers are usually chosen on the basis of size, number of reactive groups, solubility and their potential to conceal or even eliminate the anti-peptide response. The link between the peptide and the carrier is chosen in such a way that the structure of the peptide is not perturbed or shielded by the carrier. In general, peptides incorporating epitopes in multiple copies with branched architecture are better immunogens than linear oligomers and were found to be superior in eliciting antibodies when compared to the same determinants assembled in a linear conformation. Moreover, formulation of an effective immunogen that could elicit a potent immune response should incorporate B-cell epitopes that mimic the three-dimensional conformation of the antigen as well as T-cell epitopes resulting from the processed protein, both on the same carrier that links the MHC with the T-cell receptor. To achieve enhanced and prolonged humoral and cellular immune response, potent immunogens must be also administered in combination with external immunoadjuvants. The LCP system containing the 2-amino-dodecanoic lipoamino acids C_{12} (H_2N–$CH(CH_2)_9CH_3$–COOH) was found to be a potent immunogen with self-adjuvanting properties, eliciting humoral and cellular responses irrespective of the administration route. This α-amino acid with its long alkyl side chain combines structural features of lipids with those of amino acids and peptides, and when conjugated to or incorporated into peptides or drugs enhances the passage of the pharmacologically active compounds across biological membranes. The design of our peptide-based vaccine was meant to combine the LCP system with branched lysines orthogonally protected with several peptides of different sequences and lengths. These sequences are epitopes of glycoproteins involved in the mechanism of virus entry in host cells. The potential T- and B-cell epitopes were chosen using computational programs based on hydrophobicity, solvent accessibility and secondary structure. The T-cell epitopes were synthesized in order to allow a possible secondary structure longer than the predicted sequences for gB493–509 gB577–599 epitopes and gD119–140 gD215–234. B-cell epitopes were synthesized in short sequences to avoid synthetic difficulty. In fact the N^{α} terminal positions can be hindered by steric constrain of previously coupled peptides. Peptide conjugates were obtained using a combination of different peptide solid phase strategies. Branched polypeptide conjugates were obtained using a convergent strategy. An advantage of the convergent strategy is chemical unambiguity because the protected peptide segments and the branching unit used in coupling reactions are purified prior to the reaction, thereby limiting the range of by-products and facilitating purification. Using this strategy we were able to conjugate three different epitopes on the LCP core.

Stimulating U937 and RAW 264.7 cells with LCP constructs induced significant MAPKs activation. This pathway of signal transmission co-operates with other pathways of gene activation to the synthesis of IL-6, IL-8 or MIP-2 and TNF-α. Many viruses can use as specific receptors, macrophage or lymphocyte surface molecules modifying the antiviral functions of these cells.[52,53] Wang *et al*. demonstrated that a SARS-surface viral protein stimulates the production of TNF-α and IL-6.[55]

These results provide evidence that the LCP constructs initiate macrophages activation, leading to the production of pro-inflammatory cytokines. LCP complexed with immunodominating viral peptides enhances the signal transduction pathways with a predominant activation of the ERK1/2 pathway. A combination of Lys_3-LCP with non-covalent mixtures of both gB and gD has been analysed in signal transduction pathway assays to evaluate cytokine production showing results that almost superimpose results obtained using the Lys_3-LCP construct alone, thus confirming the relevance of the covalent link between peptide epitopes and the lipidic construct to obtain optimal activation.

The cells implicated in the immunological response contain a large repertoire of receptors which are responsible for surface interaction with microorganisms and their components. These cell surface interactions lead to activation of several signal transmission pathways which induce cytokine synthesis and release, therefore, our results show that LCP constructs bearing HSV gB or gD derived peptides represent a promising system for the production of efficient immunogens.

Therefore, the idea of selecting the best HSV epitopes from gB and gD for the development of a multivalent (LCP) vaccine might be of great benefit and a practical way to generate broader multiepitope protective T-cell responses.

Abbreviations

APC	Antigen presenting cells
Boc	*tert*-Butyloxycarbonyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DIEA	Diisopropylethylamine
DMF	*N,N*-Dimethylformamide
ERK	Extracellular signal Regulated protein Kinase
FACE	Fast activated cell-based
ELISA	INT, Tetrazolium salt
HATU	2-(7-Aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-Hydroxy-1,2,3-benzotriazole
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
IL-6	Interleukin-6
IL-8	Interleukin-8
INT	(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride)
JNK	Jun NH_2-terminal kinase
LAL	Limulus amoebocyte lysate
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MIP-2	Macrophage inflammatory peptide-2
PAM	Phenylacetamidomethyl-resin
Pam_3Cys	*N*-Palmitoyl-*S*-[2,3-bis(palmitoyloxy-(2*R*,*S*)-propyl]-(*R*)-cysteine
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RP-HPLC	Reverse-phase high-pressure liquid chromatography
RPMI 1640	Roswell Park Memorial Institute medium
SPPS	Solid phase peptide synthesis
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid
TIS	Triisopropylsilane

References

1 R. Arnon, Synthetic peptides as the basis for vaccine design, *Mol. Immunol*., 1991, **28**, 209–215.
2 L. BenMohamed, S. L. Wechsler and A. B. Nesburn, Lipopeptide vaccines-yesterday, today, and tomorrow, *Lancet Infect. Dis*., 2002, **2**, 425–431.
3 J. A. Berzofsky, J. D. Ahlers and I. M. Belyakov, Strategies for designing and optimizing new generation vaccines, *Nat. Rev. Immunol*., 2002, **3**, 209–219.
4 M. Singhand and D. T. O'Hagan, Recent advances in vaccine adjuvants, *Pharm. Res*., 2002, **19**, 715–728.
5 S. L. Elliott, S. Pye, T. Le, L. Mateo, J. Cox, L. Macdonald, A. A. Scalzo, C. A. Forbesand and A. Suhrbier, Peptide based cytotoxic T-cell vaccines; delivery of multiple epitopes, help, memory and problems, *Vaccine*, 1999, **17**, 2009–2019.
6 R. K. Gupta and G. R. Siber, Adjuvants for human vaccines-current status, problems and future prospects, *Vaccine*, 1995, **13**, 1263–1276.
7 I. Toth, M. Danton, N. Flinnand and W. A. Gibbons, A combined adjuvant and carrier system for enhancing synthetic peptides immunogenicity utilising lipidic amino acids, *Tetrahedron Lett*., 1993, **34**, 3925–3928.
8 P. M. Moyle and I. Toth, Self-adjuvanting lipopeptide vaccines, *Curr. Med. Chem*., 2008, **15**, 506.
9 C. Olive, I. Tothand and D. J. Jackson, Technological advances in antigen delivery and synthetic peptide vaccine developmental strategies, *Mini-Rev. Med. Chem*., 2001, **1**, 429–438.
10 C. Olive, M. Batzloff, A. Horváth, T. Clair, P. Yarwood, I. Toth and M. F. Good, Group A streptococcal vaccine delivery by immunization with a self-adjuvanting M protein-based lipid core peptide construct, *Indian J. Med. Res*., 2004, **119**, 88–94.
11 K. Deres, H. Schild, K. H. Wiesmüller, G. Jung and H. G. Rammensee, *In vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopepide vaccine, *Nature*, 1989, **342**, 561–564.
12 A. Mora and J. Tam, Controlled lipidation and encapsulation of peptides as a useful approach to mucosal immunizations, *J. Immunol*., 1998, **161**, 3616–3623.
13 B. Nardelli, B. Paul, H. P. James and J. Tam, Oral administration of an antigenic synthetic lipopeptide (MAP-P3C) evokes salivary antibodies and systemic humoral and cellular responses, *Vaccine*, 1994, **12**, 1335–1339.
14 W. Zeng, D. C. Jackson, J. Murray, K. Rose and L. E. Brown, Totally synthetic lipid-containing polyoxime peptide constructs are potent immunogens, *Vaccine*, 2000, **18**, 1031–1039.
15 L. BenMohamed, H. Gras-Masse, A. Tartar, P. Daubersies, K. Brahimi, M. Bossus, A. Thomas and P. Druilhe, Lipopeptide immunization without adjuvant induces potent and long-lasting B, T helper, and cytotoxic T lymphocyte responses against a malaria liver stage antigen in mice and chimpanzees, *Eur. J. Immunol*., 1997, **27**, 1242–1253.
16 G. Zong, I. Toth, R. Reid and R. C. Brunham, Immunogenicity evaluation of a lipidic amino acid-based synthetic peptide vaccine for Chlamydia trachomatis, *J. Immunol*., 1993, **151**, 3728–3736.
17 I. Toth, N. Flinn, W. A. Gibbons, M. Good, W. Hayman and F. Brown, *Immunological evaluation of the lipid-core-peptide (LCP) adjuvant/carrier system*, in *Peptides Chemistry, Structure and Biology*, ed. T. P. Pravin, R. Kaumaya and S. Hodges, Mayflower Scientific Ltd., Kingswinford, 1996, pp. 810–811.
18 C. Olive, M. R. Batzloff, A. Horváth, A. Wong, T. Clair, P. Yarwood, I. Toth and M. F. Good, A lipid core peptide construct containing a conserved region determinant of the group A streptococcal M protein elicits heterologous opsonic antibodies, *Infect. Immun*., 2002, **70**, 2734–2738.
19 C. Olive, M. Batzloff, A. Horváth, T. Clair, P. Yarwood, I. Toth and M. F. Good, Potential of lipid core peptide technology as a novel self-adjuvanting vaccine delivery system for multiple different synthetic peptide immunogens, *Infect. Immun*., 2003, **71**, 373–2383.
20 C. Olive, K. Hsien, A. Horváth, T. Clair, P. Yarwood, I. Toth and M. F. Good, Protection against group A streptococcal infection by vaccination with self-adjuvanting lipid core M protein peptides, *Vaccine*, 2005, **23**, 298–2303.
21 C. Olive, M. F. Ho, J. Dyer, D. Lincoln, N. Barozzi, I. Toth and M. F. Good, Immunization with a tetraepitopic lipid core peptide vaccine construct induces broadly protective immune responses against group A streptococcus, *J. Infect. Dis*., 2006, **193**, 1666–1676.
22 W. A. Hayman, I. Toth, N. Flinn, M. Scanlon and M. F. Good, Enhancing the immunogenicity and modulating the fine epitope recognition of antisera to a helical group A streptococcal peptide vaccine candidate from the M protein using lipid-core peptide technology, *Immunol. Cell Biol*., 2002, **80**, 178–187.
23 E. de Oliveira, J. Villen, E. Giralt and D. Andreu, Synthetic Approaches to Multivalent Lipopeptide Dendrimers Containing Cyclic Disulfide Epitopes of Foot-and-Mouth Disease Virus, *Bioconjugate Chem*., 2003, **14**, 144–152.
24 J. Tam and Y. Lu, A. Vaccine engineering: enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T-and B-cell epitopes, *Proc. Natl. Acad. Sci. U. S. A*., 1989, **86**, 9084–9088.
25 Y. Haro, S. Perez, M. Garcia, W. C. Chan and G. Ercilla, Liposome entrapment and immunogenic studies of a synthetic lipophilic multiple antigenic peptide bearing VP1 and VP3 domains of the hepatitis A virus: a robust method for vaccine design, *FEBS Lett*., 2003, **540**(1–3), 133–140.
26 C. J. Fitzmaurice, L. E. Brown, T. L. McInerney and D. C. Jackson, The assembly and immunological properties of non-linear synthetic immunogens containing T-cell and B-cell determinants, *Vaccine*, 1996, **14**, 553–560.

27 S. Depil, O. Moralès, F. A. Castelli, N. Delhem, V. François, B. Georges, F. Dufossé, F. Morschhauser, J. Hammer, B. Maillère, C. Auriault and V. Pancré, Determination of a HLA II promiscuous peptide cocktail as potential vaccine against EBV latency II malignancies, *J. Immunother*., 2007, **30**, 215–226.
28 L. Shen, R. Schroers, J. Hammer, X. F. Huang and S. Y. Chen, Identification of a MHC class-II restricted epitope in carcino-embryonic antigen, *Cancer Immunol. Immunother*., 2004, **53**, 391–403.
29 J. Hammer, T. Sturniolo and F. Sinigaglia, HLA class II peptide binding specificity and autoimmunity, *Adv. Immunol*., 1997, **66**, 67–100.
30 W. W. Kwok, J. A. Gebe, A. Liu, S. Agar, N. Ptacek, J. Hammer, D. M. Koelle and G. T. Nepom, Recent Progress in Herpes Simplex Virus Immunobiology and Vaccine Research, *Trends Immunol*., 2001, **22**, 583–588.
31 L. BenMohamed, G. Bertrand, C. D. McNamara, H. Gras-Masse, J. Hammer, S. L. Wechsler and A. B. Nesburn, Identification of Novel Immunodominant CD^+ Th1-Type T-Cell Peptide Epitopes from Herpes Simpex Virus Glycoprotein D That Confer Protective Immunity, *J. Virol*., 2003, **77**, 9463–9473.
32 B. Rost and C. Sander, Prediction of protein secondary structure at better than 70% accuracy, *J. Mol. Biol*., 1993, **232**, 584–589.
33 P. Prevelige and G. D. Fasman, *Prediction of Protein Structure and Principles of Protein Conformation*, Plenum Press, New York, 1990.
34 S. Smith and N. J. Robinson, Age-Specific Prevalence of Infection with Herpes Simplex Virus Types 2 and 1: A Global Review, *J. Infect. Dis*., 2002, **186**(Suppl 1), 3–28.
35 D. I. Bernstein and L. R. Stanberry, Herpes simplex virus vaccines, *Vaccine*, 1999, **171**, 1681–1689.
36 M. Aymard, Current epidemiology of herpes, *Pathol. Biol*., 2002, **50**, 425–435.
37 R. J. Whitley, D. W. Kimberlin and B. Roizman, Herpes simplex viruses, *Clin. Infect. Dis*., 1998, **26**, 541–553.
38 S. S. Cush, K. M. Anderson, D. H. Ravneberg, J. L. Weslow-Schmidt and E. Flano, Memory Generation and Maintenance of CD8+ T Cell Function during Viral Persistence, *J. Immunol*., 2007, **179**, 141–153.
39 A. A. Chentoufi, X. Zhang, K. Lamberth, G. Dasgupta, I. Bettahi, A. Nguyen, M. Wu, X. Zhu, A. Mohebbi, S. Buus, S. L. Wechsler, A. B. Nesburn and L. BenMohamed, HLA-A *0201-Restricted $CD8^+$ Cytotoxic T Lymphocyte Epitopes Identified from Herpes Simplex Virus Glycoprotein D, *J. Immunol*., 2008, **180**, 426–437.
40 P. Y. Chou and G. D. Fasman, Prediction of protein conformation, *Biochemistry*, 1974, **13**, 222–245.
41 P. Y. Chou and G. D. Fasman, Conformational parameters for amino acids in helical, b-sheet and random coil regions calculated from proteins, *Biochemistry*, 1974, **13**, 211–222.
42 M. Galdiero, A. Tortora, N. Damiano, M. Vitiello, A. Longanella and E. Galdiero, Induction of cytokine mRNA expression in U937 cells by *Salmonella typhimurium* porins is regulated by different phosphorylation pathways, *Med. Microbiol. Immunol*., 2005, **194**, 13–23.
43 M. F. Taylor, J. D. Paulauskis, D. D. Weller and L. Kobzik, In vitro efficacy of morpholino-modified antisense oligomers directed against tumor necrosis factor-alpha mRNA, *J. Biol. Chem*., 1996, **271**(29), 17445–17452.
44 A. Cuenda, J. Rouse, Y. N. Doza, R. Meier, P. Cohen, T. F. Gallagher, P. R. Yang and J. C. Lee, SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1, *FEBS Lett*., 1995, **364**, 229–233.
45 D. R. Alessi, A. Cuenda, P. Cohen, D. T. Dudley and A. R. Saltiel, PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*, *J. Biol. Chem*., 1995, **270**, 27489–27494.
46 C. Bonny, A. Oberson, S. Negri, C. Sauser and D. F. Schorderet, Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death, *Diabetes*, 2001, **50**, 77–82.
47 E. T. Yin, C. Galanos, S. Kinsky, R. A. Bradshaw, S. Wessler, O. Luderity and M. E. Sarmiento, Picogram sensitive assay for endotoxin: gelation of *Limulus polyphemus* blood cell lysate induced by purified lipopolysaccharide and lipid A from Gram negative bacteria, *Biochim. Biophys. Acta*, 1972, **261**, 284–289.
48 I. Bettahi, A. B. Nesburn, S. Yoon, X. Zhang, A. Mohebbi, V. Sue, A. Vanderberg, S. L. Wechslerand and L. BenMohamed, Protective immunity against ocular herpes infection and disease induced by highly immunogenic self-adjuvanting glycoprotein D lipopeptide vaccines, *Invest. Ophthalmol. Visual Sci*., 2007, **48**, 4643–4653.
49 A. B. Nesburn, T. V. Ramos, X. Zhu, H. Asgarzadeh, V. Nguyen and L. BenMohamed, Local and systemic B cell and Th1 responses induced following ocular mucosal delivery of multiple epitopes of herpes simplex virus type 1 glycoprotein D together with cytosine-phosphate-guanine adjuvant, *Vaccine*, 2005, **23**, 873–883.
50 W. A. Gibbons, R. A. Hughes, M. Charalambous, M. Christodoulou, A. Szeto, A. E. Aulabough, P. Mascagni and I. Toth, Lipidic Peptides I. Synthesis resolution and structural elucidation of fatty amino acids and their homo- and hetero-oligomers, *Liebigs Ann. Chem*., 1990, 1175–1183.
51 M. Krishna and H. Narang, The complexity of mitogen-activated protein kinases (MAPKs) made simple, *Cell. Mol. Life Sci*., 2008, **65**, 3525–3544.
52 J. Garcia, B. Lemercier, S. Roman-Roman and G. Rawadi, A Mycoplasma fermentans-derived synthetic lipopeptide induces AP-1 and NF-kappaB activity and cytokine secretion in macrophages *via* the activation of mitogen-activated protein kinase pathways, *J. Biol. Chem*., 1998, **273**, 34391–34398.
53 S. R. Paludan, J. Melchjorsen, L. Malmgaard and S. C. Mogensen, Expression of genes for cytokines and cytokine-related functions in leukocytes infected with Herpes simplex virus: comparison between resistant and susceptible mouse strains, *Eur. Cytokine Network*, 2002, **13**, 306–316.
54 J. Kelly, M. H. Beddall, D. Yu, S. R. Iyer, J. W. Marsh and Y. Wu, Human macrophages support persistent transcription from unintegrated HIV-1 DNA, *Virology*, 2008, **372**, 300–312.
55 W. Wang, L. Ye, L. Ye, B. Li, B. Gao, Y. Zeng, L. Kong, X. Fang, H. Zheng, Z. Wu and Y. She, Up-regulation of IL-6 and TNF-alpha induced by SARS-coronavirus spike protein in murine macrophages *via* NF-kappaB pathway, *Virus Res*., 2007, **128**, 1–8.