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In vivo antigen loading and activation of dendritic cells via a liposomal peptide vaccine mediates protective antiviral and anti-tumour immunity

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Abstract

Initiation of antiviral and anti-tumour T cell responses is probably achieved mainly by dendritic cells (DC) transporting antigen from the periphery into organised lymphoid tissues. To develop T cell vaccines it is, therefore, important to understand the accessibility of the antigen to DC in vivo and whether DC are activated by vaccination. Here we have evaluated the immunogenicity of a liposomal vaccine formulation with antigenic peptides derived from the glycoprotein of the lymphocytic choriomeningitis virus. Liposome-encapsulated peptides were highly immunogenic when administered intradermally and elicited protective antiviral immunity. After intradermal injection, liposomes formed antigen depots which facilitated long-lasting in vivo antigen loading of dendritic cells almost exclusively in the local draining lymph nodes. The immunogenicity of the liposomal peptide vaccine was further enhanced by incorporation of immunostimulatory oligonucleotides leading to activation of DC. This optimised liposomal peptide vaccine elicited also anti-tumour immunity and induced CTL responses comparable to adoptively transferred, peptide-presenting DC. Thus, our data show that liposomal formulations of peptide vaccines are highly effective at direct in vivo antigen loading and activation of DC leading to protective antiviral and anti-tumour immune responses.

Keywords: Antigenic peptides; Vaccination; Tumour immunity

1. Introduction

Immunity against many viruses and tumours is mediated by CTL recognition of MHC class I-associated peptides on the target cell and lysis of tumour or virus-infected cells in a contact-dependent, perforin-mediated fashion [1,2]. Recent technical advances have facilitated the rapid characterisation of antigens and immunogenic peptides derived from tumours [3,4], viruses, or other microbial pathogens [5]. Thus, pathogen- or tumour-specific synthetic peptide vaccines can be generated readily and are being used in clinical trials to induce CTL-mediated antiviral or anti-tumour immunity [6,7]. However, high avidity binding of an immunodominant peptide to the respective MHC class I molecule is not the only factor which makes a peptide a good vaccine. Whereas protective, CTL-mediated antiviral immunity can be induced by immunisation using immunodominant epitopes simply mixed with IFA [8,9] or immunostimulatory oligonucleotides [10], peptide immunisation may have the adverse effect of inducing peptide-specific tolerance when administered systemically and repetitively at high doses

Abbreviations: DC, dendritic cells; LCMV, lymphocytic choriomeningitis virus.

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[8,11,12]. There is evidence that the local distribution of the peptide is the critical factor affecting the immunogenicity of a peptide vaccine [8,12]. Thus, since immune responses are initiated exclusively in organised lymphoid tissues [13], immunogenic peptide formulations should, therefore, be designed to facilitate localised release and transport to organised lymphoid tissues whilst keeping systemic distribution to a minimum.

Dendritic cells (DC) are the key APC population mediating antigen transport to organised lymphoid tissues and initiation of T cell responses [14]. The quality of a peptide vaccine is, therefore, probably determined by the efficacy and duration of peptide loading of DC in vivo. In vitro loading of DC with peptides and/or proteins is efficient, and adoptive transfer of such antigen-loaded DC in the mouse has been shown to mediate protection both against tumours [15,16] and viral infections [17]. Furthermore, DC-immunisation may induce or enhance anti-tumour responses in human cancer patients [18,19]. However, in these treatment protocols, autologous DC are usually cultured ex vivo, loaded with antigen and reinfused into the patient. Such procedures are expensive, laborious and potentially accompanied by undesired side-effects. Therefore, it is important to evaluate alternative approaches facilitating in vivo antigen-delivery to dendritic cells.

The use of liposomes as antigen carriers has the advantages of simplicity, safety and being relatively inexpensive [20]. Liposomes also appear particularly well suited to target antigens to dendritic cells: (a) after peripheral subcutaneous application, liposomes distribute preferentially via the lymph and reach local organised lymphoid tissues [21], (b) protein encapsulated in pH-sensitive liposomes reach DC in vivo and induce primary CTL responses [22,23], (c) the surface of liposomes may be specifically modified to optimise binding to DC, for example, via specific sugar moieties [24] or antibodies [25].

In this study, we evaluated the immunogenicity, in vivo distribution and bioavailability for DC of a liposomal peptide vaccine using immunodominant epitopes derived from the glycoprotein of the lymphocytic choriomeningitis virus (LCM-GP).

2. Materials and methods

2.1. Mice

All mice were obtained from the Institut für Labortierkunde (University of Zürich, Switzerland) at the age of 8–16 weeks and were sex-matched. Transgenic mice expressing the LCMV-GP33 epitope in all tissues (H8 mice) have been previously described [26]. Mice transgenic for a Vα2/Vβ8.1 TCR specific for H2-D^b and the major LCMV-GP epitope, GP33 (318 mice) [11] and mice transgenic for a Vα2/Vβ8.1 TCR specific for I-A^b and the LCMV-GP epitope P13 (Smarta mice) [27], were used as donors of transgenic T cells. All animals were kept under SPF conditions.

2.2. Viruses and cell lines

LCMV-WE strain, originally obtained from Dr. F. Lehmann-Grube (Hamburg, Germany), was propagated on L929 cells. EL-4 (H-2^b) thymoma cells were used as target cells in cytotoxicity assays. EL-4 cells stably transfected with a construct expressing a GP33-H-$2K^b$ fusion protein under the control of the H-$2K^b$ promotor have been generated in our laboratory (Ochsenbein et al.).

2.3. Peptides and oligonucleotides

LCMV peptides GP33 [11] and P13 [27] were purchased from Neosystem Laboratoire (Strasbourg, France). The immunostimulatory oligonucleotide ODN1668 (5′-TCCATGACGTTCCTGATGCT-3′) was synthesised by Microsynth (Balgach, Switzerland).

2.4. Preparation of liposomes

GP33 was labeled with BODIPY FL,SE (Molecular Probes, Eugene, OR) for the determination of encapsulated peptide in the liposomes. 1 mg GP33 dissolved in 0.3 ml bicarbonate (pH 8.0) and 2.5 mg BODIPY FL,SE (50 mg/ml) were incubated at room temperature for 2 h. The reaction was stopped by addition of 30 μl hydroxylamine (1.5 M, pH 8.5). After 1 h the mixture was eluted with phosphate buffer (PB, 67 mM, pH 7.4) on a Biogel P6-DG column (30 × 1 cm) to remove unreacted dye. Labeled GP33 was used as tracer in mixtures with known amounts of unlabeled peptide. Small unilamellar liposomes were prepared by freeze-thawing followed by sequential filter extrusion. The basic composition of the liposomes used was 200 mg/ml soy phosphatidylcholine (SPC), 25 mg/ml cholesterol and 1.20 mg/ml DL-α-tocopherol. The dried lipid mixture was solubilised with GP33 (4 mg/ml), P13 (4 mg/ml) and/or ODN1668 (250 nmol/ml), subjected to 3–5 freeze-thaw cycles, and repetitively extruded through Nuclepore (Sterico AG, Dietikon, Switzerland) filters (0.8, 0.4 and 0.2 μm pore size). Unencapsulated peptides and oligonucleotides were removed by dialysis and the amount of encapsulated peptide determined by fluorescence measurement at 513 nm. Liposome size and homogeneity was determined by laser light scattering (Submicron Particle Sizer Model 370, Nicomp, Santa Barbara, USA) resulting in homogeneous populations of unilamellar liposomes with a size range of 80–180 nm. Liposomes

were filter sterilised and diluted in BSS (balanced salt solution). Peptide encapsulation was in a range between 80% and 90% as determined by fluorescence of the BODIPY FL,SE-labeled peptides. Liposome-encapsulated peptides were injected at the indicated concentrations i.p. in a volume 200–500 μl, directly into the spleen in a volume of 25 μl, i.v. via the tail vein (200–500 μl), s.c. in the flank (2 depots of 50 μl), and i.d. in the flank (4 depots of 25 μl).

2.5. Preparation of dendritic cells

Generation of dendritic cells from C57BL/6 and H8 bone marrow cultures has been previously described [17]. DC express high amounts of the costimulatory molecules CD80, CD86, and CD40 and >50% of the cells express high levels of MHC class II antigens. For peptide pulsing, DC were resuspended in RPMI/5% FCS at 10^6 ml^{-1} and incubated at 37°C for 60 min. DC were washed two times with BSS and intravenously injected in a volume of 0.5 ml. To enrich for lymph node dendritic cells, lymph nodes were ground through a sterile stainless steel screen with a syringe plunger. Cells were resuspended in RPMI 1640/5% FCS and antibiotics at 2×10^7 ml^{-1} and low density cells were separated using metrizamide (14.5% in RPMI 1640/5% FCS, Sigma) gradient centrifugation. Dendritic cell suspensions showed a purity of 60–70% as determined by FACS analysis using anti-CD11c staining (Pharmingen). Activation of lymph node DC was detected by two colour flow cytometry using anti-CD11c-FITC together with biotinylated anti-CD54 or anti-CD86 (all Pharmingen) followed by incubation with streptavidin-Tricolor (Caltag). The cell suspensions were analysed on a FACScan flow cytometer (Becton Dickinson).

2.6. Cytotoxicity assays

Spleen cells (4×10^6 $well^{-1}$) from primed mice were restimulated for 5 days in 24-well tissue culture plates with 2×10^6 GP33-labeled, irradiated (1500 rad) spleen cells in IMDM/10% FCS. Restimulated spleen effector cells from one well were resuspended in 1 ml MEM/2% FCS and three-fold serial dilutions were made (indicated as dilution of culture). EL-4 cells were labeled with GP33 (10^{-6} M) and 250 μCi ^{51}Cr for 1.5 h at 37°C. Target cells (10^4 $well^{-1}$) were incubated for 4.5 h in 96-well round-bottom plates with spleen effector cells. EL-4 cells without peptide served as controls. Spontaneous release was always below 19%.

2.7. Viral titers and protection against LCMV

Naive C57BL/6 mice were immunised with the liposomal peptide vaccine or with dendritic cells. Eight days later, mice were challenged i.v. with 200 pfu of LCMV. Virus titers in the spleens were determined 4 days after i.v. challenge in a LCMV infectious focus assay as previously described [28].

2.8. Proliferation assay

Spleen cells (2×10^5) from TCR transgenic 318 or Smarta mice were incubated in round-bottom 96-well plates with three-fold serial dilutions of lymph node DC, starting with 2×10^4 DC/well. After incubation for 60 h at 37°C/5% CO_2, [^{3}H]thymidine (1 μCi/well) was added for a further incubation period of 12 h. Proliferation was determined as [^{3}H]thymidine incorporation using a Microbeta scintillation counter (Wallac, Turku, Finland).

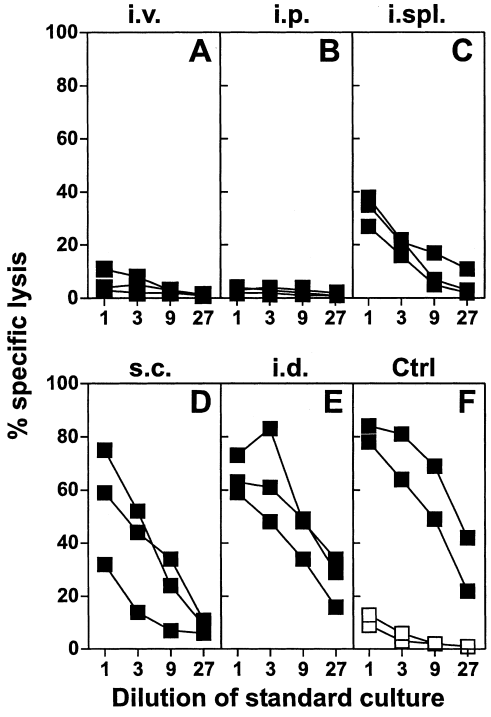

Fig. 1. Priming with liposome-encapsulated peptide GP33. Two to three C57BL/6 mice per group were immunised by intravenous (A), intraperitoneal (B), intrasplenic (C), subcutaneous (D), or intradermal (E) injection with 20 μg GP33 encapsulated in liposomes. As controls, 20 μg GP33 in IFA were injected subcutaneously (F, closed symbols) or 20 μg GP33 in PBS was injected intradermally (F, open symbols). Eight days later induction of GP33-specific CTL was tested. Spleen cells were restimulated in vitro for 5 days with peptide-labeled, irradiated spleen cells. Specific lysis was measured on GP33-labeled EL-4 target cells. Unspecific lysis of EL-4 cells without peptide was <10%. Results shown are from one out of three comparable experiments.

3. Results

3.1. High immunogenicity of liposome-encapsulated peptides after intradermal injection

In the first set of experiments we evaluated whether GP33 encapsulated in liposomes elicits CTL responses comparable to the IFA-peptide formulation and tested the influence of the application route on the immunogenicity of the liposomal vaccine. Intravenous (Fig. 1(A)) or intraperitoneal (Fig. 1(B)) injection of 20 μg GP33 encapsulated in liposomes did not elicit a significant GP33-specific CTL response. In contrast, direct injection into the spleen (Fig. 1(C)) or subcutaneous (Fig. 1(D)) application induced GP33-specific cytotoxicity. Most efficient was the intradermal application route (Fig. 1(E)) leading to CTL responses comparable to the s.c. injection of GP33 in IFA (Fig. 1(F), closed symbols). Intradermal injection of GP33 as free peptide did not induce a CTL response (Fig. 1(F), open symbols), indicating that the encapsulation of the peptide in liposomes greatly enhanced the immunogenicity of the peptide.

3.2. Efficient CTL priming by the liposomal vaccine is enhanced by immunostimulatory oligonucleotides and T helper peptides

Next, we sought to further optimise the liposomal peptide vaccine and to test the ability of the vaccine to induce protective antiviral immunity. Oligonucleotides containing unmethylated CpG oligodinucleotides (CpG-ODNs) have been shown to act as an adjuvant for protein and peptide vaccines [10,29]. Here, we encapsulated both GP33 and the CpG-ODN 1668 (ODN1668) in liposomes and tested whether ODN1668 improves the immunogenicity of the liposomal vaccine. Intradermal application of GP33 in liposomes induced strong CTL responses over a broad concentration range

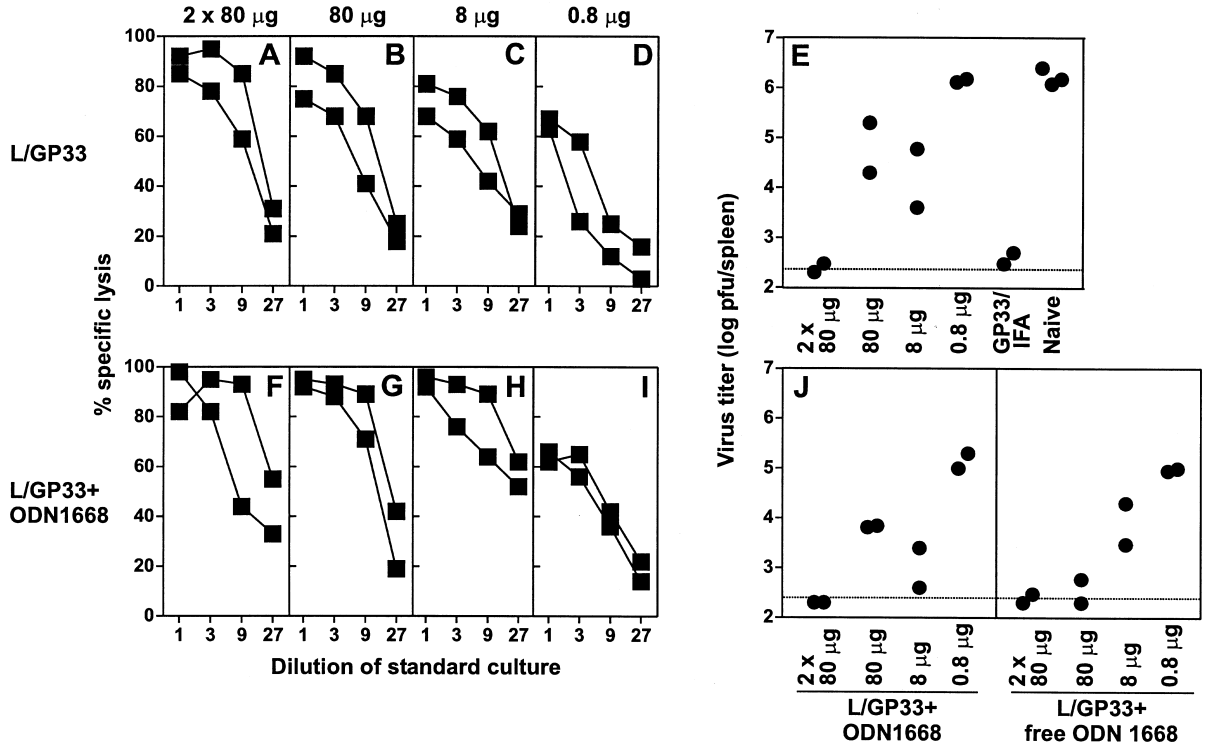

Fig. 2. Titration of liposome-encapsulated GP33 and adjuvant effect of the CpG oligodinucleotide 1668 (ODN1668) on CTL induction and protection against LCMV challenge infection. Graded doses of liposome-encapsulated GP33 (A)–(E) or GP33 encapsulated together with ODN1668 (F)–(J) were injected i.d. into naive C57BL/6 mice either once on day 0 or where indicated twice (2×) on days 0 and 2. On day 8 spleen cells were restimulated in vitro for 5 days with peptide-labeled, irradiated spleen cells and cytotoxicity was determined on GP33-labeled EL-4 target cells (A)–(D) and (F)–(I). Each line represents values from one mouse. Unspecific lysis of EL-4 cells without peptide was always $<10\%$. Alternatively, protection against LCMV-WE was tested at day 8 post immunisation (E) and (J). Virus titer in spleens was determined 4 days after i.v. challenge with 200 pfu LCMV-WE. The detection limit is represented by the dotted line. Mice immunised s.c. with 80 μg GP33 in IFA and naive, untreated mice served as controls (E). The amount of ODN1668 incorporated in liposomes (L/33 + ODN1668 in (F)–(I) and left panel of (J)) was estimated from the incorporation rate of GP33 (10 nmol for 80 μg GP33, 1 nmol for 8 μg GP33, and 0.1 nmol for 0.8 μg GP33). Alternatively, GP33 containing liposomes were mixed with 10 nmol ODN1668 (L/GP33 + free ODN1668 in right panel of (J)). One of the two identical experiments is shown.

(Fig. 2(A)–(D)). This treatment elicited complete protection against i.v. challenge infection with LCMV when applied in high doses (two i.d. immunisations with 80 μg), or partial protection when applied in intermediate doses (one immunisation with 80 μg or 8 μg) (Fig. 2(E)). Incorporation of ODN1668 together with GP33 into liposomes enhanced the immunogenicity of the peptide vaccine leading to enhanced GP33-specific CTL responses (Fig. 2(F)–(I)) and improved protection against LCMV challenge infection (Fig. 4(J), left panel). The adjuvant effect of incorporated ODN1668 (Fig. 2(J), left panel) was comparable to non-incorporated ODN1668 mixed with the GP33 liposomes; both elicited similar GP33-specific CTL responses (data not shown) and efficient protection against LCMV infection (Fig. 2(J), right panel).

Concomitant T help is an important cofactor for optimal CTL responses [30]. To test the effect of the simultaneous liposomal delivery of a T helper peptide on GP33-specific CTL responses, we encapsulated GP33 together with the LCMV-GP peptide P13 [27] in liposomes. Table 1 shows that P13 was successfully encapsulated in liposomes together with GP33 leading to efficient long-lasting loading of dendritic cells in draining lymph nodes. Moreover, as with ODN1668, simultaneous incorporation of GP33 and P13 enhanced GP33-specific CTL responses (Fig. 3(A)–(D)). An enhancing effect of P13 on the antiviral protection was difficult to evaluate in these experiments, since GP33-liposomes alone induced good antiviral protection (Fig. 3(E)). Taken together, these results show that, firstly, combinations of immunogenic peptides and adjuvant compounds such as immunostimulatory ODN can be incorporated in liposomes, and, secondly, these combinations improve the immunogenicity of the liposomal peptide vaccine.

3.3. Induction of anti-tumour CTL responses by the liposomal peptide vaccine

We next tested the ability of the liposomal peptide formulation to induce anti-tumour immunity. To generate an optimised liposomal peptide vaccine, we encapsulated the CTL epitope GP33 together with the Th epitope P13 and ODN1668 into liposomes. Tumour cells used in this set of experiments were EL-4 thymoma cells expressing GP33 as the only H-2^b-restricted LCMV-GP epitope (EL4-GP33). Small pieces of EL4-GP33 tumours ($2 \times 2 \times 2$ mm) containing $2–5 \times 10^6$ cells were implanted subcutaneously into naive recipients and therapeutic treatment was started 1 day after transplantation. Mice were either immunised i.d. with different doses of liposome-encapsulated peptides plus ODN1668 (Fig. 4(A)–(C)), injected s.c. with constitutively GP33-expressing DC (H8-DC) (Fig. 4(D)) or left untreated (Fig. 4(E)). The transplanted tumours grew progressively in untreated controls (Fig. 4(E)) and were only partially controlled in mice immunised with 1 μg of the vaccine (Fig. 4(C)). Importantly, a single immunisation with 100 μg (Fig. 4(A)) or 10 μg (Fig. 4(B)) of the liposomal peptide vaccine mediated complete control of the tumour, comparable to s.c. immunisation with H8-DC (Fig. 4(D)). Thus, the liposomal peptide vaccine induced both protective antiviral and anti-tumour immunity.

To further assess the quality of the liposomal peptide vaccine, we compared the in vivo CTL-induction of DC constitutively expressing GP33 (H8-DC), either injected i.v. (Fig. 5(A)) or s.c. (Fig. 5(B)), or C57BL/6-DC exogenously pulsed with GP33 (B6-DC/GP33) (Fig. 5(C)) with the optimised liposomal formulation (Fig. 5(D)). Eight days after immunisation, spleen cells were harvested and cytotoxicity was determined after secondary restimulation for 5 days with GP33-pulsed,

Table 1
Duration of peptide delivery to dendritic cells in draining lymph nodes after intradermal injection of liposome-encapsulated versus peptides in aqueous solution

Time post i.d. injection (h)	Proliferation of naive TCR transgenic T cells after in vivo loading of lymph node DC (cpm $\times 10^{-3}$)[a]			
	Liposomal peptides		Peptides in solution	
	318 CTL	Smarta Th cells	318 CTL	Smarta Th cells
24	474 ± 10	383 ± 22	277 ± 10	275 ± 14
48	399 ± 7	269 ± 13	55 ± 7	71 ± 6
72	116 ± 4	62 ± 5	28 ± 5	19 ± 3

[a] Naive C57BL/6 mice were immunised i.d. either with liposomes containing 100 μg GP33 and P13, and 10 nmol ODN1668 or the same amount of peptide and ODN1668 solubilised in 100 μl PBS. Pooled lymph node DC from draining lymph nodes of three mice per group and day were used as stimulators in a proliferation assay using either 318 or Smarta spleen cells as resonders (DC to responder ratio was 1:20). Values represent mean of cpm $\times 10^{-3}$ ±SD. Control cpm values were for unpulsed DC: 35.7 ± 1.9 for 318 responder cells, 15.6 ± 1.6 for Smarta responder cells; GP33-pulsed DC: 461.5 ± 10.7 for 318 responder cells; P13-pulsed DC: 399.9 ± 10.9 for Smarta responder cells.

irradiated spleen cells. Immunisation with the liposomal peptide vaccine (Fig. 5(D)) induced CTL activities comparable to peptide-presenting DC (Fig. 5(A)–(C)), confirming the excellent immunogenicity of the liposomal peptide vaccine.

3.4. Prolonged exogenous in vivo loading and activation of dendritic cells by the liposomal peptide vaccine

To clarify the mechanisms by which the liposomal peptide vaccine elicits such potent CTL responses, we tested firstly the distribution pattern of GP33 on DC in different lymph nodes. Liposomes containing 100 μg GP33 were injected i.d. into the left flank of naive C57BL/6 mice and DC were isolated 24 h later either from the draining lymph nodes (inguinal, axillary, and brachial) of the same or of the opposite side. Peptide-loading of DC was assessed in a proliferation assay by co-cultivation with CD8+ T cells from TCR transgenic 318 mice recognising GP33 presented on H-2D^b [11] (Fig. 6). Comparable to DCs pulsed in vitro, lymph node DC pulsed in vivo isolated from the side of injection fully activated TCR transgenic 318 cells (Fig. 6(A)), and were comparable with DC pulsed with GP33 in vitro (Fig. 6(B)) indicating that DC from the local draining lymph nodes had been efficiently loaded via the liposomal peptide vaccine in vivo. Lymph node DC from the opposite side (right flank) stimulated 318 cells only very weakly (Fig. 6(A)) showing that just a few DC in non-draining lymph nodes were peptide loaded. Injection of liposomes i.d. containing GP33 and ODN1668 resulted in the same distribution pattern with almost exclusive loading of DC from the local draining lymph nodes (data not shown).

Next, we assessed the duration of peptide presentation by DC after peptide delivery via liposomes. To this end, 50 μg GP33 and P13 plus ODN1668 were injected i.d. in both flanks of naive C57BL/6 mice either liposome-encapsulated or in aqueous solution (Table 1). At the indicated time points lymph node DC were isolated and used as stimulators in a proliferation assay. Twenty-four hours after injection of liposome-encapsulated or free peptides, lymph node DC were efficiently loaded with both GP33 and P13 leading to strong proliferation of the peptide-specific 318 CTL and Smarta T helper cells, although the in vivo antigen loading via liposomes appeared to be slightly better (Table 1). Importantly and in contrast to DC from mice immunised with free peptides, DC from liposome-immunised mice retained an efficient stimulatory capacity also after 2 and 3 days (Table 1). This suggests that the liposomal formulation results in sustained release of peptides from the local injection site facilitating long-lasting peptide-loading of DC in the draining lymph nodes.

Finally, we tested whether the liposomal vaccine mediates activation of DC with upregulation of costimulatory and/or adhesion molecules. Eighteen hours after i.d. injection of liposomes containing either 50 μg GP33 (Fig. 7(A) and (C)) or 50 μg GP33 plus ODN1668 (Fig. 7(B) and (D)), DC from the local draining lymph nodes where isolated and expression of the adhesion molecule CD54 (Fig. 7(A) and (B)) and the costimulatory molecule CD86 (Fig. 7(C) and (D))

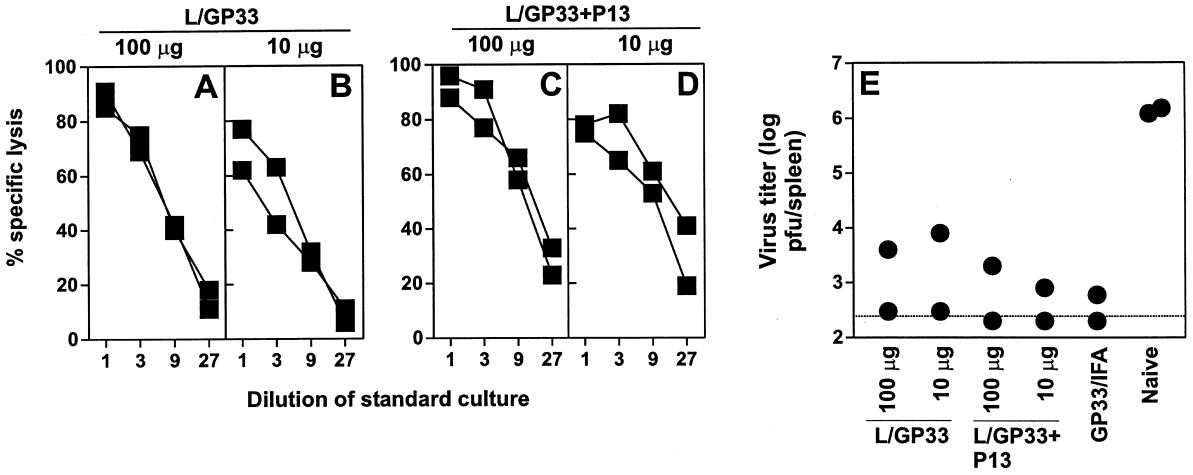

Fig. 3. Influence of simultaneous incorporation of the T helper epitope P13 on CTL induction and protection against LCMV challenge infection by the liposomal GP33-formulation. The indicated doses of liposome-encapsulated GP33 ((A), (B) and (E)) or GP33 encapsulated together with P13 ((C)–(E)) were injected i.d. into naive C57BL/6 mice on day 0. Eight days later, spleen cells were restimulated in vitro for 5 days and cytotoxicity was determined as in Fig. 1 on GP33-labeled EL-4 target cells ((A)–(D)). Each line represents values from one mouse. Unspecific lysis of EL-4 cells without peptide was <9%. Protection against LCMV-WE was tested at day 8 post immunisation (E). Virus titer in spleens was determined 4 days after i.v. challenge with 200 pfu LCMV-WE. Detection limit is represented by the dotted line. Mice immunised s.c. with 100 μg GP33 in IFA and naive, untreated mice served as controls (E). One of two experiments with comparable results is shown.

was determined by flow cytometry. After immunisation with liposomes containing GP33, DC in the draining lymph nodes did not significantly upregulate both CD54 (Fig. 7(A)) or CD86 (Fig. 7(C)). However, i.d. delivery of GP33 plus ODN1668 via liposomes led to an activation of DC with considerable upregulation of CD86 (Fig. 7(D)) and a minor shift in the expression of CD54 (Fig. 7(B)); thus the improved immunogenicity of the liposomal peptide vaccine containing ODN1668 correlated with in vivo activation and maturation of DC. Taken together, the good immunogenicity of the liposomal vaccine may be due to a combinatorial effect of a local antigen depot leading to a prolonged, localised release of immunogenic peptides and, when combined with immunostimulatory compounds, mediating activation of DC in regional lymphoid tissues.

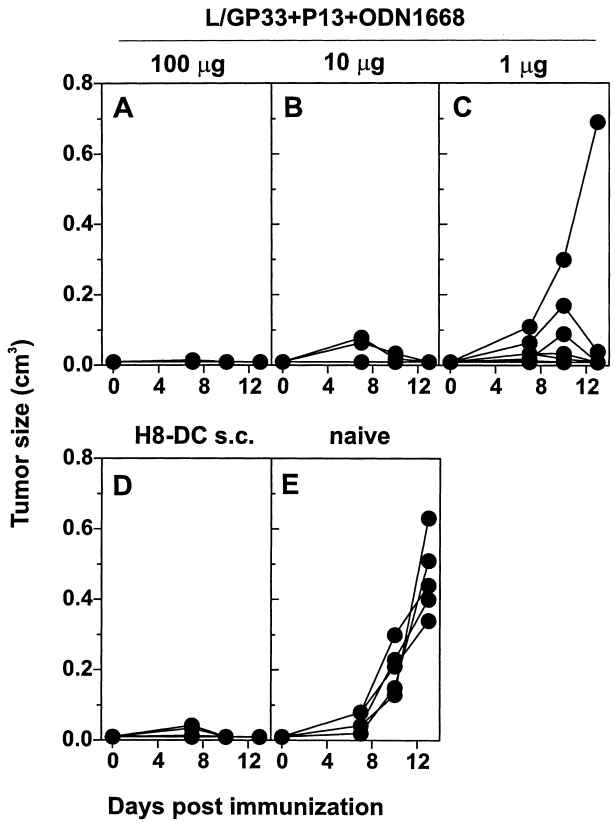

Fig. 4. Induction of tumour immunity via liposome-encapsulated peptides. Small tumour pieces of EL4-GP33 tumours were implanted s.c. in the flank of naive C57BL/6 mice. One day later (day 0) the liposomal peptide vaccine was injected i.d. containing the indicated amount of the GP33 and P13, and 10, 1, or 0.1 nmol ODN1668 (A)–(C), 2×10^5 constitutively GP33-expressing H8-DC were injected s.c. (D), or mice were left untreated (E). Tumour size was assessed at the indicated time point post immunisation. Tumour volume was calculated by the formula $V = \pi x\ abc/6$, where a, b and c are the orthogonal diameters. Results from one of two comparable experiments are shown.

4. Discussion

In this study, we have shown that localised in vivo loading of DC with antigenic peptides is efficiently achieved by intradermal immunisation with a liposomal vaccine formulation. This simple method of liposomal peptide vaccination achieved excellent functional responses, elicited protective anti-tumour and antiviral immunity and induced CTL responses were comparable to ex vivo manipulated, peptide-pulsed DC.

Characterisation of immunodominant epitopes derived from pathogen or tumour antigens facilitates efficient use of various synthetic antigenic peptides to prime or boost anti-tumour or antiviral immune responses specifically. However, the efficacy of a peptide vaccine is crucially determined by the dose and route of application (Ref. [8] and this report). Peptide immunisation may impair immune responses against subsequent tumour [12] or viral [8] challenge and it has been suggested that such tolerising effects may be caused by the rapid systemic distribution of the peptide resulting in presentation by too many non-professional APC within and outside lymphoid organs [12]. However, peptide-induced tolerisation can be avoided and protective tumour immunity induced when the peptide is presented by DC [31]. Therefore, the formulation and application of a peptide vaccine should first avoid systemic distribution of the peptide and secondly facilitate efficient presentation of the peptide by dendritic cells. The liposomal peptide formulation described in this report fulfils these criteria. Liposome-encapsulated GP33 was highly immunogenic when administered intradermally and provided excellent protective antiviral immunity even after repetitive immunisation. Long-lasting in vivo antigen loading of dendritic cells, almost entirely restricted to the local draining lymph nodes, was facilitated by the sustained release of peptide-containing liposomes from the intradermal depot. Altogether, our data corroborate and significantly extend previous reports showing that immunisation with liposomes is a practical approach to deliver antigen to DC in vivo [22,23]. However, these studies did not evaluate functional protection against tumours or viruses, nor has the in vivo distribution of the peptide on DC been followed.

CTL responses are significantly improved in the presence of cognate T help [30]. Accordingly, simultaneous delivery of T helper epitopes has increased the efficacy of CTL peptide vaccines [32] facilitating induction of protective tumour immunity also against MHC class II negative tumours [33]. However, the requirements for concomitant T help for the induction of CTL responses can be reduced by adjuvants that activate dendritic cells leading to enhanced CTL activation [34–36]. These findings may explain the high immunogenicity of the combined liposomal peptide vaccine

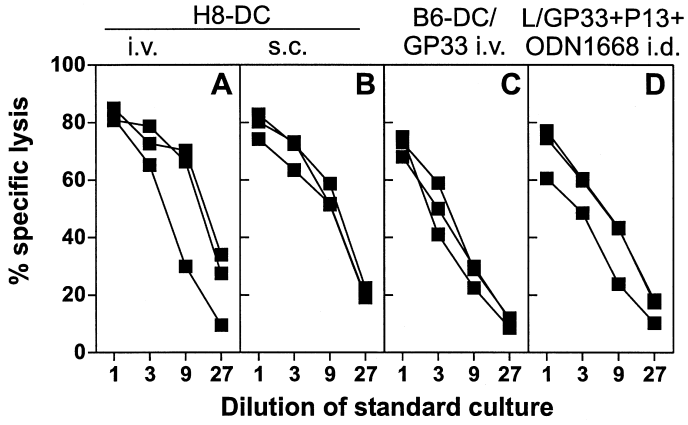

Fig. 5. Comparison of CTL induction after immunisation with peptide-presenting DC and liposome-encapsulated peptides. Naive C57BL/6 mice received 2×10^5 H8-DC either i.v. (A) or s.c. (B), 2×10^5 B6-DC exogenously pulsed with GP33 i.v. (C), or liposomes containing the 100 μg GP33 and P13, and 10 nmol ODN1668 i.d. (D). On day 8, spleen cells were restimulated in vitro for 5 days and cytotoxicity was determined as in Fig. 1 on GP33-labeled EL-4 target cells. Each line represents values from one mouse. Unspecific lysis of EL-4 cells without peptide was $<9\%$. Representative data from one out of three experiments are shown.

containing GP33 together with the Th epitope P13 and ODN1668. DC continuously loaded with GP33 and P13 from the liposomal peptide depot probably simultaneously activate both specific CTL and Th cells, thereby amplifying the CTL response. CpG-ODN can activate DC in vitro [37] and, as shown here, the delivery of ODN1668 in the liposomal peptide vaccine activated DC in vivo. Therefore, the simultaneous delivery of Th epitopes and CpG-ODN probably favoured the generation of a microenvironment in the local lymph nodes that facilitated the efficient induction of GP33-specific CTL capable of mediating anti-tumour and antiviral effector functions.

The ability to induce efficient tumour immunity using the combined liposomal peptide vaccine is relevant to current clinical approaches aimed to elicit anti-tumour immune responses. These exploit the excellent immunogenicity of DC, but often involve expensive and laborious ex vivo-manipulations to load DC with antigen [18,19]. Thus, direct in vivo antigen loading and activation of DC via liposomal vaccines would be an easy and practical alternative to these protocols.

Liposomal vaccines may also be further improved by modifications that specifically mediate DC targeting. For example, Fc-fragments of antibodies directed against certain cell surface molecules can be coupled to liposomes facilitating specific interactions with the target cell. Serre et al. showed that targeting of liposome-encapsulated antigen to DC in vitro may be augmen-

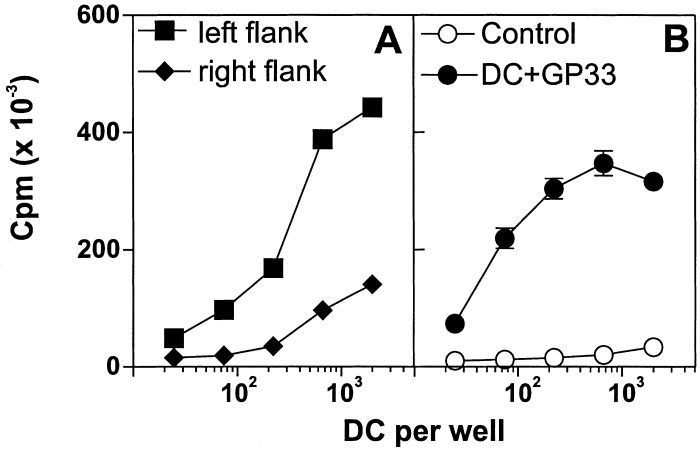

Fig. 6. Distribution of GP33 as determined by in vivo antigen loading of lymph node DC. (A) 100 μg liposome-encapsulated GP33 was injected i.d. into the left flank. 24 h later DC from the draining lymph nodes from the same side (left flank) or the opposite side (right side) were isolated and the proliferative responses of naive TCR transgenic CD8+ T cells from 318 mice were determined. (B) Lymph DC from the injected side of liposome-treated mice exogenously pulsed with GP33 served as positive control. Lymph node DC from naive C57BL/6 mice were used to monitor non-specific activation. T cell proliferation was measured by ^{3}H-thymidine incorporation. One of the two comparable experiments is shown.

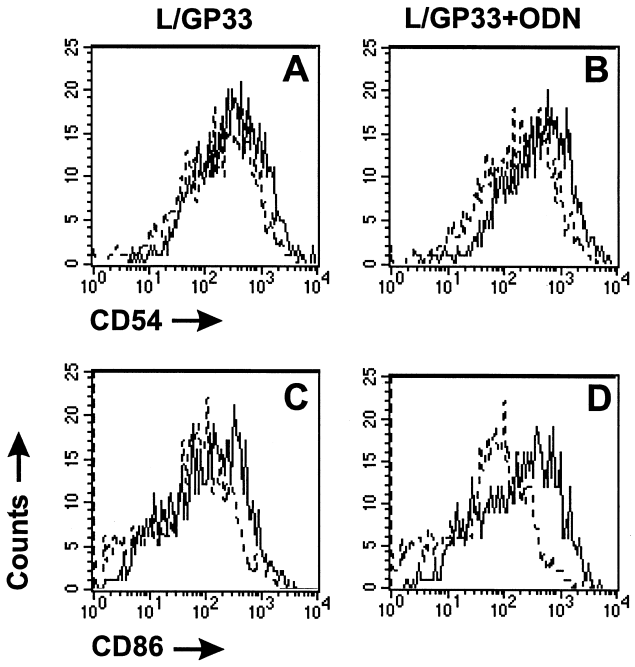

Fig. 7. Activation of DC in local draining lymph nodes after immunisation with liposome-encapsulated peptides. Flow cytometric analysis of the expression of CD54 ((A) and (B)) or CD86 ((C) and (D)) on CD11c-positive DC 18 h after i.d. immunisation with 50 μg GP33 in liposomes (solid line in (A) and (C) or 50 μg GP33 plus ODN1668 in liposomes (solid line in (B) and (D)). The dotted line indicates staining of DC from naive control animals.

ted >1000-fold by simple antibody modifications of the liposomes [25]. Furthermore, since DC specifically recognise carbohydrate moieties of pathogens and use these pattern recognition receptors to internalise large amounts of antigen [38], specific targeting of antigen-loaded liposomes to these receptors might increase the immunogenicity of liposomal vaccines. For example, the modification of a liposomal anti-HIV vaccine with different oligomannose residues elicited a good adjuvant effect [24] that may be due to improved delivery of the antigen to DC.

In summary, the findings presented in this report, in concert with previous data [22,23], clearly indicate that liposomal antigen delivery to DC in vivo appears to be a promising approach to induce efficient antiviral and anti-tumour immune responses. It appears likely that such a delivery system together with an improved and specific targeting to dendritic cells may be well suited to elicit strong immune responses mediating protection against viral infections or rapidly growing tumours.

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