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The novel chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA_3-DTDA) promotes stable binding of His-tagged proteins to liposomal membranes: Potent anti-tumor responses induced by simultaneously targeting antigen, cytokine and costimulatory signals to T cells

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Abstract

Recent studies indicate that the chelator lipid nitrilotriacetic acid ditetradecylamine (NTA-DTDA) can be used to engraft T cell costimulatory molecules onto tumor cell membranes, potentially circumventing the need for genetic manipulation of the cells for development of cell- or membrane-based tumor vaccines. Here, we show that a related lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA_3-DTDA, which has three NTA moieties in its headgroup instead of one) is several-fold more effective than NTA-DTDA at promoting stable His-tagged protein engraftment. IAsys biosensor studies show that binding of His-tagged B7.1 (B7.1-6H) to NTA_3-DTDA-containing membranes, exhibit a faster *on-rate* and a slower *off-rate*, compared to membranes containing NTA-DTDA. Also, NTA_3-DTDA-containing liposomes and plasma membrane vesicles (PMV) engrafted with B7.1-6H and CD40-6H exhibit greater binding to T cells, in vitro and in vivo. Engrafted NTA_3-DTDA-containing PMV encapsulated cytokines such as IL-2, IL-12, GM-CSF and IFN-γ, allowing targeted delivery of both antigen and cytokine to T cells, and stimulation of antigen-specific T cell proliferation and cytotoxicity. Importantly, use of B7.1-CD40-engrafted PMV containing IL-2 and IL-12 as a vaccine in DBA/2J mice induced protection against challenge with syngeneic tumor cells (P815 mammary mastocytoma), and regression of established tumors. The results show that stable protein engraftment onto liposomal membranes using NTA_3-DTDA can be used to simultaneously target associated antigen, costimulatory molecules and cytokines to T cells in vivo, inducing strong anti-tumor responses and immunotherapeutic effect.

Keywords: Chelator lipid; T cell; Costimulatory molecule; Cytokine; Tumor vaccine; Liposome; Plasma membrane vesicle

1. Introduction

The transfection of tumor cells to induce expression of T cell costimulatory molecules and/or cytokines, is a potentially viable approach for the development of cell-based tumor vaccines and immunotherapies [1–3]. Protective immunity has notably been reported following vaccination of animals with tumor cells that have been transfected to express different costimulatory and adhesion molecules such as B7.1 (CD80), B7.2 (CD86), CD40, and ICAM-1 [4–8], and different immunostimulatory cytokines such as GM-CSF, IL-2 and IL-12 [9–12]. The use of genetically modified tumor cells as vaccines, however, is impractical, and may present safety concerns for widespread clinical use.

Of importance to the development of immunotherapies is the fact that, when used as a vaccine or adjuvant, cytokine that is associated with liposomal structures can often exhibit enhanced efficacy and reduced toxicity, compared to "free" cytokine [13–15]. For example, liposome-associated IL-2 was found to be several orders of magnitude more potent at promoting survival of the murine T cell clone D10 in vitro [16], and mice vaccinated

Abbreviations: PMV, Plasma membrane vesicles; Streptavidin-FITC, Fluorescein isothiocyanate-conjugated streptavidin; POPC, Palmitoyl-oleoyl-phosphatidylcholine; DMPC, Dimyristoyl-phosphatidylcholine; NTA-DTDA, nitrilotriacetic acid ditetradecylamine; PEG_{400}, Polyethyleneglycol-400; FITC-DHPE, *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine, triethylammonium salt; PE-PEG_{2000}, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene-glycol-2000

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with irradiated tumor cells exhibit a more potent anti-tumor response and less toxicity, if IL-2 is co-administered as a liposome-associated, rather than as a "free" or soluble form [17–20]. Interestingly, the delivery of cytokines to the site of antigen presentation also is purported to enhance systemic immune protection [21,22]. The targeting of antigenic, costimulatory and cytokine-mediated signals to T cells in vivo, therefore, could well have synergistic effects in inducing tumor immunity.

In previous studies, we showed that recombinant forms of the costimulatory molecules B7.1 and CD40 bearing a histidine tag can be anchored or "engrafted" onto the surface of liposomes and tumor cell-derived plasma membrane vesicles (PMV), following incorporation of the chelator-lipid nitrilotriacetic acid ditetradecylamine (NTA-DTDA). The engrafted molecules enable such modified membranes to be targeted to T cells through the cognate receptor interactions CD86-CD28 and CD40-CD40L [16,23]. To further develop this approach, in the present work, we report the properties of a novel chelator-lipid, NTA_3-DTDA, which is related to NTA-DTDA, but contains three NTA moieties in its headgroup instead of one (see Fig. 1). IAsys biosensor studies show that His-tagged proteins bind more strongly to membranes containing NTA_3-DTDA than to membranes containing NTA-DTDA. When incorporated into P815 cell-derived PMV, NTA_3-DTDA enabled stable engraftment of B7.1 and CD40. Engrafted PMV containing IL-2, IL-12, GM-CSF and IFN-γ allowed targeted delivery of these cytokines to T cells and induced effective anti-tumor responses when used as vaccines.

2. Materials and methods

2.1. Reagents

Paraformaldehyde was obtained from BDH Chemicals (Kilsyth, Victoria, Australia). RPMI 1640 medium was obtained from Gibco-BRL (Invitrogen, Melbourne, Australia). Fetal calf serum (FCS) was obtained from Trace, Biosciences (Noble Park, Victoria, Australia). Sulfo-NHS-LC-Biotin and fluorescein isothiocyanate-conjugated streptavidin (streptavidin-FITC) was obtained from Pierce (Rockford, IL). Dimyristoyl-phosphatidylcholine (DMPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), β-mercaptoethanol and polyethylene glycol-400 (PEG_{400}) were obtained from Sigma-Aldrich (Castle Hill, N.S.W., Australia). *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethyl-ammonium salt (FITC-DHPE) was from Molecular Probes (Eugene, OR), and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene-glycol-2000 (PE-PEG_{2000}) was from Avanti Polar Lipids Inc. The chelator-lipid 3(nitrilotriacetic acid)-ditetradecy-

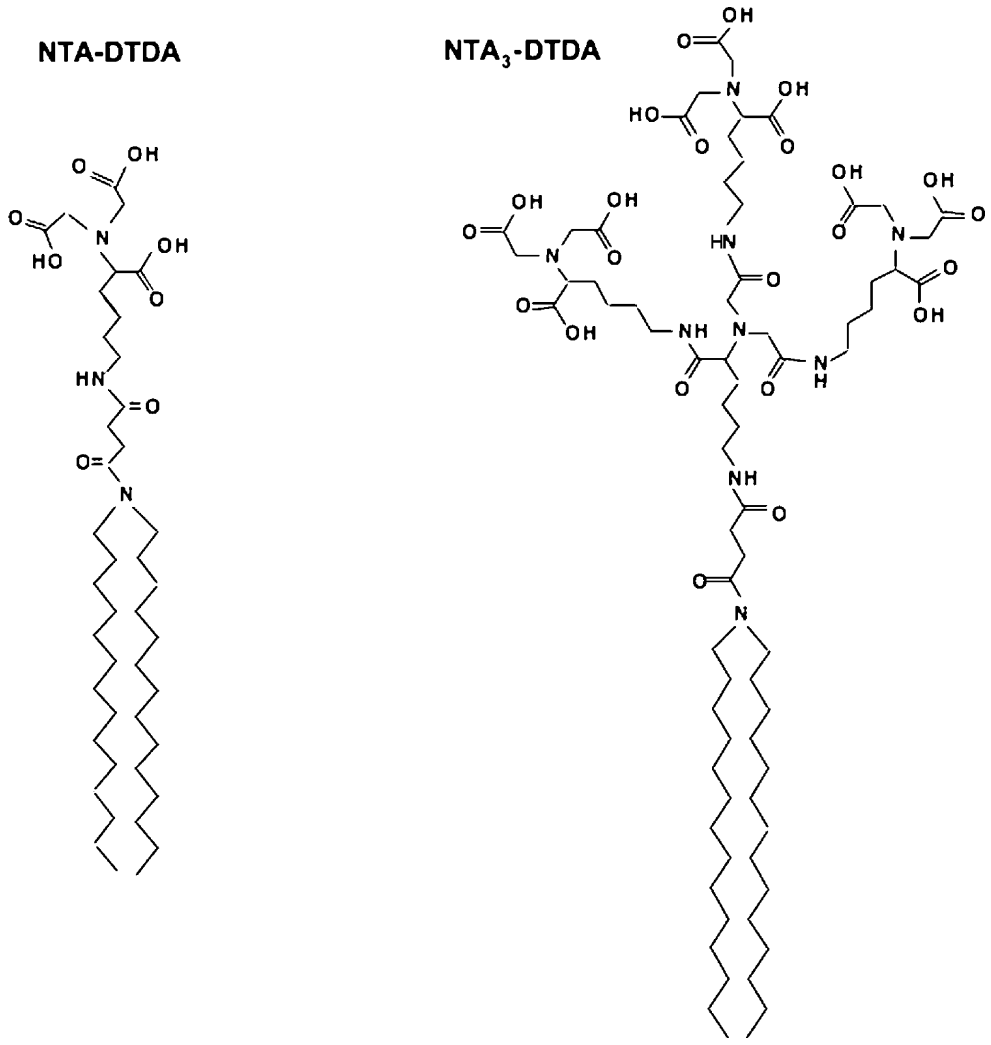

Fig. 1. The structure of NTA (nitrilotriacetic acid) ditetradecylamine (NTA-DTDA) and 3NTA (nitrilotriacetic acid) ditetradecylamine (NTA_3-DTDA).

lamine (NTA_3-DTDA), consisting of three nitrilotriacetic acid (NTA) head groups covalently linked to two ditetradecylamine (DTDA) chains was synthesized essentially as described [24], but with additional steps to covalently couple a NTA group onto each carboxyl group of the NTA-DTDA, to produce NTA_3-DTDA (J.G. Altin, manuscript in preparation). $NiSO_4$ was used for all additions of Ni^{2+} to buffers.

2.2. Mice and cells

Female or male DBA/2J mice (H-2^d), were used for isolation of splenic T cells for use in binding assays using flow cytometry, for T cell proliferation and cytotoxicity assays, and for vaccinations and assessment of tumor growth in vivo. The mice were used at 6–8 weeks of age and were obtained from the Animal Breeding Establishment, John Curtin School of Medical Research (JCSMR), Australian National University (ANU), Canberra. For isolation of splenic T cells, spleens were removed and dissociated into single cell suspension, the red cells were removed by hypotonic lysis and centrifugation, and the T cells then purified by nylon wool adherence, essentially as described [16].

Murine P815 mastocytoma [DBA/2J (H-2^d)] cells were obtained from Dr. H. Warren (John Curtin School of Medical Research, ANU). The murine $CD4^+$ T cell clone D10 [AKR/J (H-2^k)] specific for conalbumin, was obtained from Dr. B. van Leeuwen (School of Biochemistry and Molecular Biology, ANU). All cells were cultured in complete medium consisting of RPMI 1640 containing 10% FCS and 2 mM glutamine, and maintained at 37 °C in an incubator with an atmosphere of 5% CO_2. The D10 cells also were routinely grown in the presence of 50 IU/ml murine IL-2 as described [25]. Expression of CD28 and CD40L on D10 cells was upregulated by pre-incubation of the cells with the TCR activation idiotype-specific mAb 3D3 (5 μg/ml) for 18–24 h, as previously described [23].

2.3. Recombinant proteins

Recombinant human IL-2 (Proleukin) was obtained from EuroCetus (Amsterdam, The Netherlands). Recombinant murine IL-12, IFN-γ and GM-CSF were obtained from PeproTech Inc. (Rockey Hill, NJ). Where indicated, recombinant cytokines were biotinylated by reacting with sulfo-NHS-LC-biotin as described [25]. Recombinant forms of the extracellular regions of murine B7.1 (CD80) and CD40, denoted B7.1-6H, and CD40-6H, respectively; and the extracellular region of the human erythropoietin receptor (EPOR), denoted EPOR-6H, each with a hexahistidine (6H) tag at the carboxy terminal, were expressed using the baculovirus expression system with the proteins being purified from the culture supernatants of virus-infected High-5 cells as described [26]. Recombinant proteins were stored at −20 °C in PBS at a concentration of 0.2–0.6 mg/ml; the proteins were thawed at 37 °C and vortexed gently prior to use in each experiment.

2.4. IAsys biosensor studies

Membranes containing DMPC, POPC and either NTA-DTDA or NTA_3-DTDA as indicated, were deposited onto the IAsys sensing surface of an IAsys biosensor dual-cell cuvette from a suspension of liposomes exactly as described [24]. After membrane deposition, the cuvette was washed 4 times with PBS and 4 times with distilled water, before again washing with PBS and allowing the instrument to equilibrate for 5–10 min to achieve a stable baseline before use in binding studies. Binding experiments were carried out at 25 °C in PBS using the indicated test membrane in one cell and a control membrane containing 95 mol% DMPC and 5 mol% POPC in the other cell. The contents of each cell were stirred continuously (80 cycles/s) and binding to the sensing surface in each cell, was measured at 2 s intervals in units of arc seconds (arc s). Proteins were added to the test cell from stock solutions made in PBS to give the appropriate final protein concentration. Binding of B7.1-6H to membranes was monitored for 5 min; the contents of each cell were then replaced by washing 4 times with PBS, and dissociation then monitored for 20 min. For analysis of binding data, the signal of the control cell was subtracted from that of the test cell to remove the bulk refractive index change and the effect of any non-specific binding.

Kinetic constants were evaluated from profiles for the binding of B7.1-6H to membranes containing either NTA-DTDA or NTA_3-DTDA using the "Fast Fit" program. k_{obs} was calculated from a single exponential fit of the binding phase, and k_{on} was determined from the gradient of the plot of k_{obs} versus protein concentration which approximated a straight line. As a single exponential dissociation did not adequately fit the dissociation data, and k_{off} when determined from the y-intercept of the plot of k_{obs} versus protein concentration was $\sim 10^{-3}$, as suggested by the manufacturer, therefore, double exponential fitting was used to calculate the *fast* and slow k_{off} components. The dissociation constant was determined using the expression $K_d = k_{off}/k_{on}$. Other details have been described [24].

2.5. Plasma membrane vesicles

2.5.1. Preparation

PMV were prepared from murine P815 mastocytoma as described [16,27]. Briefly, cultured P815 cells (1×10^8) were washed twice with PBS, and the cells were suspended in homogenization buffer [10 mM sodium phosphate (pH 7.4) containing 30 mM NaCl, 1 mM $MgCl_2$, and 0.02% NaN_3], and homogenized by brief sonication at 4 °C. The cell lysate was then layered over a solution of 41% sucrose and then centrifuged (95,000×*g*) for 1 h at 4 °C. The PMV were collected from the interfacial band and washed twice in homogenization buffer by centrifugation (95,000×*g*, 20 min, 4 °C). Stock PMV suspensions were stored at −20 °C, and briefly re-sonicated prior to use in each experiment.

2.5.2. Incorporation of NTA_3-DTDA and cytokine

Liposomes were prepared from a mixture of POPC, NTA-DTDA and FITC-DHPE (in molar ratio of 78:20:2), or POPC, NTA_3-DTDA, and FITC-DHPE (in molar ratio of 96:2:2) dissolved in ethanol/chloroform (3:1). For some experiments, the tracer lipid BODIPY-PC (Molecular Probes) was used instead of FITC-DHPE; BODIPY-PC has a higher quantum yield and was found to give a higher level of cell fluorescence following the binding of liposomes (not shown). For functional studies, the tracer lipid was omitted from the lipid mixtures. Lipid mixtures were dried under a stream of nitrogen gas, and liposomes were produced by rehydrating the desiccated lipids in 100 μl PBS containing Ni^{2+} (200 μM Ni^{2+} for mixtures containing NTA-DTDA and 60 μM Ni^{2+} for mixtures containing NTA_3-DTDA), and suspending to a total lipid concentration of 1 mM. Where indicated, cytokines were incorporated with liposomes by rehydrating the lipids in 100 μl PBS containing 50 ng IL-2, IL-12, GM-CSF or IFN-γ, in addition to Ni^{2+}. Liposome suspensions (with or without cytokines) were then produced as described [16]. The resulting liposomes were then fused with PMV by mixing 100 μl of the indicated liposome suspension with 100 μl of PMV (1×10^7 cell equivalents); and then adding 15% PEG_{400}, and immediately diluting the composite vesicles in PBS. For PMV containing cytokine, the vesicles were purified by size exclusion chromatography on Sephadex columns (G25 for PMV containing IL-2, GM-CSF and IFN-γ, and G100 for PMV containing IL-12) (Pharmacia Biotech, Uppsala, Sweden) with PBS, to remove any unincorporated cytokine.

To assess the efficiency of incorporation of cytokines into the modified PMV, the cytokines were biotinylated and then associated with liposomes and fused with PMV (as described above). The modified PMV were then subjected to size-exclusion chromatography, and both the vesicle-incorporated and unincorporated-cytokine fraction were eluted and collected separately. Each fraction was then concentrated using a Centricon-10 (Millipore, Bedford, MA) microconcentrator, and run on SDS-PAGE (5–15% acrylamide gel) under reducing conditions. The biotinylated proteins were then electrophoretically transferred onto a nitrocellulose membrane, and detected by enhanced chemiluminescence (ECL) after probing the membrane with streptavidin-HRP as described [25].

2.5.3. Engraftment of costimulatory molecules

For engraftment of recombinant protein(s) onto the vesicle surface, purified PMV containing incorporated NTA-DTDA or NTA_3-DTDA, and with or without encapsulated cytokine(s) as indicated, were incubated with 200 μg/ml of the appropriate 6H-tagged protein in a 0.5-ml PCR tube at room temperature for 1 h.

2.5.4. Binding of engrafted plasma membrane vesicles to cells in vitro

Splenic T cells were suspended (1×10^7 cells/ml) in RPMI 1640 medium containing 10% FCS plus 10 mM HEPES (pH 7.4), and the cells then aliquoted

into a 96-well V-bottom Serocluster plate (Costar, Corning, NY) at 2×10^5 cells/well. The cells were incubated with engrafted PMV corresponding to 2×10^5 cell equivalents for 1 h on ice with frequent gentle vortexing of the plate. After the incubation, unbound vesicles were removed by washing the cells twice with PBS containing 0.1% BSA (PBS-BSA). The cells were then fixed with 2% paraformaldehyde in PBS, and analysed for FITC-fluorescence by flow cytometry, as previously described [16].

2.6. Targeting engrafted liposomes and PMV to cells in vivo

Male or female DBA/2J mice were injected in the hind footpad using a 26-gauge needle and 1 ml syringe with conventional liposomes (CL) composed of POPC, NTA_3-DTDA and BODIPY-PC (molar ratio of 98:1:1, total lipid 0.5 mM), stealth liposomes (SL) composed of POPC, NTA_3-DTDA, BODIPY-PC and PE-PEG_{2000} (molar ratio; 93:1:1:5, total lipid 0.5 mM), or modified PMV (2×10^6 cell equivalents), each engrafted with either EPOR-6H or B7.1-6H and CD40-6H. After 14 h, the mice were euthanised and the draining and non-draining popliteal lymph nodes isolated. After removal of the RBC by hypotonic lysis, the lymphocytes were stained with biotinylated CD3ε mAb and streptavidin-PE, and then examined for BODIPY and PE-fluorescence by flow cytometry using a FacsSort (Becton Dickinson) as described [26]. The results of independent experiments are presented as the mean fluorescence intensity from peak-to-peak relative to the indicated control ± the standard error of the mean (S.E.M.).

2.7. T cell proliferation and cytotoxicity assays

Purified splenic (DBA/2J) T cells were suspended in complete growth medium (2×10^4 cells/50 μl/well) and cultured for 4 days in a 96-well flat bottom plate (Cell Wells, Corning, NY) in the presence of γ-irradiated (5000 rad) P815 tumor cell-derived PMV engrafted with recombinant proteins and containing encapsulated cytokines, at 37 °C in an atmosphere of 5% CO_2. After 4 days culture, the cells were pulsed for 6–8 h with 1 μCi of [^{3}H]-thymidine per well, for measurement of T cell proliferation as described [26].

Syngeneic DBA/2J mice were immunised subcutaneously with either PBS (control) or γ-irradiated (5000 rad) P815 cell-derived PMV (2×10^5 cell equivalents) engrafted with recombinant proteins and containing encapsulated IL-2. Spleens were removed from mice 14 days after immunisation, and the T lymphocytes (effector cells) were isolated (as above) and co-cultured (at 1×10^5 cells/well) with 1×10^5 γ-irradiated (5000 rad) native P815 cells. After 5 days of co-culture, the cytolytic activity of the effector cells was assessed in a standard ^{51}Cr-release assay.

2.8. Assessment of tumor growth and regression in vivo

Mice (DBA/2J) were immunised subcutaneously with either PBS (control) or γ-irradiated syngeneic P815 cell-derived PMV (2×10^5 cell equivalents) containing engrafted recombinant proteins and encapsulated cytokines as indicated. After 14 days, the mice were challenged with 1×10^5 native P815 cells by subcutaneous injection, and tumor growth and survival was monitored with time as described [16]. For assays of tumor regression, mice were injected subcutaneously with 1×10^5 native P815 cells, and two weeks later, the mice were vaccinated three times at 1-week intervals with γ-irradiated P815 cell-derived PMV (2×10^5 cell equivalents) containing encapsulated IL-2 and IL-12 (50 ng each). Tumor size in each group of mice was monitored, and the survival time of the animals recorded.

3. Results

3.1. Kinetics of binding of B7.1-6H to membranes containing NTA-DTDA or NTA_3-DTDA

To assess the potential of the novel chelator lipid NTA_3-DTDA to be useful for the stable anchoring of proteins onto membranes, the binding of a 6His-tagged protein (B7.1-6H) to membranes containing either NTA_3-DTDA or NTA-DTDA was compared in studies with the IAsys biosensor similar to those described [24]. Preliminary experiments indicated that, when used at the same molar ratio of carrier to chelator lipid (i.e., molar ratio of carrier lipid/chelator lipid of 10:1), NTA_3-DTDA was significantly more potent than NTA-DTDA, at promoting stable binding of His-tagged proteins to membranes (not shown). This could be due to the fact that at under these conditions, the NTA_3-DTDA provides a 3-fold higher density of NTA moieties on the membrane surface, compared to NTA-DTDA (Fig. 1). To better characterise the strength of the interaction between 6His-tagged proteins with membranes containing NTA_3-DTDA, therefore, the binding of B7.1-6H to membranes on the IAsys biosensor was examined at a range of different B7.1-6H concentrations, and at a concentration of NTA_3-DTDA and NTA-DTDA in the membrane that gives the same effective density of NTA groups.

Membranes containing DMPC and POPC (control membrane), and DMPC and POPC plus 10 mol% NTA-DTDA (test membrane), were deposited separately in the cells of an IAsys biosensor dual-cell cuvette as described [24]. After equilibration of the system, a different concentration of B7.1-6H (in the range of 20–1000 nM) was added to each cell of the cuvette, and binding to each membrane was monitored with the biosensor for 5 min. Substantial binding of B7.1-6H occurred to the test membrane, but little if any binding occurred to the control membrane. The contents of the cuvette were then replaced by washing 4 times with PBS, and the dissociation phase was monitored for 20 min. The bound protein was then removed by 4 washes with 500 mM imidazole before equilibrating in PBS and adding a different concentration of B7.1-6H. Similar experiments were performed with membranes containing 3.3 mol% NTA_3-DTDA (instead of 10 mol% NTA-DTDA), deposited in the test cell. Overlayed difference plots for the binding (and dissociation) of the different concentrations of B7.1-6H to NTA-DTDA- and to NTA_3-DTDA-containing membranes as monitored with the IAsys biosensor are shown in Fig. 2A and B, respectively. It can be seen that binding of B7.1-6H to both membranes is concentration-dependent, exhibiting saturation kinetics with near-maximal binding occurring at ~1 μM and 1–2 μM B7.1-6H for membranes containing NTA-DTDA and NTA_3-DTDA, respectively. An analysis of the binding profile for each concentration of B7.1-6H using the Fast Fit program, indicated that the binding data could generally be fitted to a single exponential expression, for each type of membrane. While some deviation from the single exponential was seen at protein concentrations >200 nM (presumably reflecting re-binding and/or complex binding events) these deviations were relatively small and were considered not to significantly affect estimates of the binding constants. A plot of the observed rate constants (k_{obs}, s^{-1}) against the concentration of B7.1-6H approximated a straight line (see Fig. 2C), with the line of best fit revealing that B7.1-6H binds with an on-rate (k_{on} = slope of the plot) of $2.62 \pm 0.06 \times 10^4$ M^{-1} s^{-1} for NTA-DTDA-containing membranes, and $1.55 \pm 0.08 \times 10^4$ M^{-1} s^{-1}, for NTA_3-DTDA-containing membranes. Similarly, the line of best fit revealed the off-rate (k_{off} = intercept with

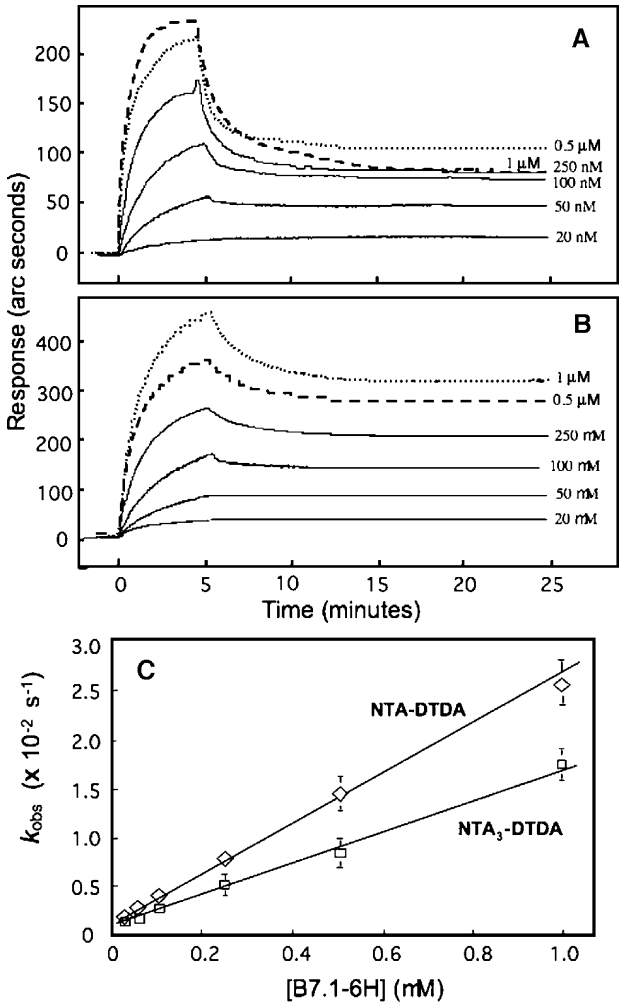

Fig. 2. Kinetics for binding of 6His-tagged proteins to membranes containing either NTA-DTDA or NTA_3-DTDA. Membranes containing DMPC and POPC (control cell), and membranes containing DMPC and POPC plus either 10 mol% NTA-DTDA or 3.3 mol% NTA_3-DTDA (test well) were each deposited in the separate wells of an IAsys biosensor cuvette. After equilibration of the system in PBS, the indicated concentration of B7.1-6H in the range of 10 nM to 1 μM was added to both the test and control wells. The binding and dissociation phase for the interaction of B7.1-6H with each membrane was then monitored with the biosensor for 5 and 20 min, respectively. Difference plots (test cell minus control profiles) for the interaction of each concentration of B7.1-6H with the NTA-DTDA-containing membrane is shown panel A, and those for the interaction of B7.1-6H with NTA_3-DTDA-containing membranes are shown in panel B. The value of k_{obs} for the binding curve for each concentration of B7.1-6H to the membranes was obtained using the linearization method (Fast Fit program), and each value for the respective membrane containing either NTA-DTDA or NTA_3-DTDA was then plotted against the concentration of protein (C). For each membrane type the plot of k_{obs} against protein concentration gives a straight line; the slope represents k_{on}, the k_{off} for the interactions were determined from the dissociation phase (see text). Error bars show the SEs from three separate experiments at each concentration.

the y-axis) for the binding of B7.1-6H to either type of membrane was $\sim 10^{-3}$ s^{-1}.

According to IAsys, k_{off} values in the range 10^{-3} to 10^{-5} s^{-1} need to be determined from the dissociation phase. Therefore, for each B7.1 concentration the k_{off} was determined by extrapolating the dissociation data to the base line by curve fitting using the Fast Fit program (not shown). At each concentration, dissociation phases were best described by two dissociation components, one having a relatively *fast* off-rate ($\sim 10^{-5}$ s^{-1}), and the other having an off-rate one or two orders of magnitude lower, especially for membranes containing NTA_3-DTDA. A summary of the on-rates, off-rates, the contribution or extent of each binding component, stability or half-life ($T_{1/2}$), and apparent dissociation constants for the interaction of B7.1-6H with NTA-DTDA and NTA_3-DTDA-containing membranes is shown in Table 1. The results indicate that the off-rates were concentration-dependent, with the least stable binding (shortest $T_{1/2}$) occurring at the highest concentration of B7.1-6H. This was more pronounced for membranes containing NTA-DTDA, than for those containing NTA_3-DTDA. The apparent dissociation constants as determined using the expression $K_d = k_{off}/k_{on}$, also were generally much lower for membranes containing NTA_3-DTDA than for membranes containing NTA-DTDA. For example, at 1 μM B7.1 the K_d were: $168 \pm 45 \times 10^{-4}$ s^{-1} (*fast*) and $4.5 \pm 0.9 \times 10^{-4}$ s^{-1} (*slow*)], and $279 \pm 37 \times 10^{-4}$ s^{-1} (*fast*) and $13.0 \pm 3.2 \times 10^{-4}$ s^{-1} (*slow*) for membranes containing NTA_3-DTDA and NTA-DTDA, respectively (Table 1). Moreover, at a B7.1-6H concentration of 1 μM, the extent of the *slow* dissociation component was ~3.5-fold greater for NTA_3-DTDA-containing membranes, compared to membranes containing NTA-DTDA. These findings indicate that B7.1-6H binds more stably and at higher levels to membranes containing NTA_3-DTDA, than to membranes containing NTA-DTDA.

3.2. Targeting plasma membrane vesicles containing either NTA_3-DTDA or NTA-DTDA

In previous studies, we showed that the chelator-lipid NTA-DTDA can be incorporated into tumor cell-derived PMV, permitting the engraftment of 6His-tagged costimulatory molecules onto vesicle surfaces for the development of tumor vaccines [16]. The greater stability of 6His-tagged proteins anchored onto membranes containing NTA_3-DTDA, compared to membranes containing NTA-DTDA, suggests that NTA_3-DTDA could have advantages for vaccine development. To explore this possibility, P815 cell-derived PMV were modified by incorporation of either NTA-DTDA or NTA_3-DTDA, and the modified PMV then engrafted with B7.1-6H and CD40-6H, before incubation with the murine T cell clone D10, to assess the binding of engrafted PMV to these cells by flow cytometry.

Preliminary binding experiments indicated that NTA_3-DTDA is also more potent than NTA-DTDA at promoting the binding of B7.1 and CD40-engrafted PMV (PMV-B7-CD40) to the murine T cell clone D10. In data not shown, near-maximal binding of engrafted PMV to D10 cells was obtained using PMV modified with POPC, NTA-DTDA and FITC-DHPE (molar ratio 78:20:2), and with POPC, NTA_3-DTDA and FITC-DHPE (molar ratio 96:2:2). PMV modified with either NTA-DTDA or NTA_3-DTDA under these conditions, and then engrafted with the control protein EPOR-6H (PMV-EPOR) and incubated with D10 cells, resulted in the fluorescence of the cells increasing to ~3-fold above background irrespective of the chelator lipid used (Fig. 3).

Table 1
Binding constants for the interaction of B7.1-6H with membranes containing either NTA-DTDA or NTA_3-DTDA

Protein (nM)	k_{on} (M^{-1} s^{-1}) × 10^4	k_{off} (s^{-1}) × 10^{-4}	Extent 9 (arc s ± 18%)	$T_{1/2}$ (min)	K_d (nM)
NTA-DTDA					
20	2.62 ± 0.06	3.6 ± 0.6 (fast)	3	31 ± 5	14 ± 3
		0.10 ± 0.03 (slow)	18	1155 ± 346	0.38 ± 0.10
50		12 ± 2 (fast)	7	9.6 ± 1.6	45 ± 8
		0.19 ± 0.03 (slow)	53	608 ± 96	0.72 ± 0.20
100		20 ± 2 (fast)	14	5.8 ± 0.6	76 ± 9
		0.41 ± 0.08 (slow)	82	281 ± 55	1.56 ± 0.34
250		39 ± 1 (fast)	73	3.0 ± 0.1	149 ± 7
		0.80 ± 0.02 (slow)	94	144 ± 4	3.0 ± 0.1
500		58 ± 3 (fast)	117	2.0 ± 0.1	221 ± 17
		1.8 ± 0.2 (slow)	108	64 ± 9	6.9 ± 1.1
1000		73 ± 8 (fast)	157	1.6 ± 0.2	279 ± 37
		3.4 ± 0.8 (slow)	88	34 ± 8	13.0 ± 3.2
NTA_3-DTDA					
20	1.55 ± 0.08	1.7 ± 0.3 (fast)	2	68 ± 12	11.0 ± 2.9
		<0.05 (slow)	42	ND	ND
50		4.6 ± 0.5 (fast)	4	25 ± 3	29.7 ± 5.6
		0.06 ± 0.01 (slow)	90	1925 ± 322	0.4 ± 0.1
100		8 ± 1 (fast)	13	14.4 ± 1.8	52 ± 11
		0.13 ± 0.02 (slow)	142	887 ± 204	0.8 ± 0.2
250		13 ± 2 (fast)	67	8.9 ± 1.3	84 ± 19
		0.28 ± 0.05 (slow)	206	412 ± 74	1.8 ± 0.4
500		20 ± 2 (fast)	122	5.8 ± 0.6	129 ± 23
		0.42 ± 0.12 (slow)	288	275 ± 80	2.7 ± 1.0
1000		26 ± 5 (fast)	148	4.4 ± 0.1	168 ± 45
		0.70 ± 0.08 (slow)	318	165 ± 18	4.5 ± 0.9

Experiments were performed by depositing membranes containing the carrier lipids DMPC and POPC plus either 10 mol% NTA-DTDA or 3.3 mol% NTA_3-DTDA into the test well of an IAsys biosensor cuvette, and then adding different concentrations of B7.1-6H in PBS (pH 7.4) (see Fig. 2). The binding and dissociation phase for the interaction of B7.1-6H with each membrane was monitored with the biosensor for 5 and 20 min, respectively (as described in the legend to Fig. 2); and kinetic constants were then determined by applying the "Fast Fit" program to the data (see text). The on-rates (k_{on}) were obtained form the slope of the line of best fit after plotting the binding data (k_{obs}) for the interaction of B7.1-6H with membranes containing either NTA-DTDA or NTA_3-DTDA, against the B7.1-6H concentration (see Fig. 1C); off-rates (k_{off}) were determined from the dissociation phase of the indicated interaction by fitting the data to a double exponential model which calculated the k_{off} and dissociation extent (for extrapolation to the baseline) for the *fast* and *slow* components of the dissociation phases. The apparent dissociation constant (K_d) for each interaction was then determined using the relationship $K_d = k_{off}/k_{on}$. Each constant represents the mean ± S.E.M.s obtained from 3 to 5 independent experiments; ND (Not determined).

Compared to control cells, however, D10 cells incubated with PMV-B7-CD40 containing NTA-DTDA exhibited a 17.5-fold increase in fluorescence intensity, whereas cells incubated with PMV-B7-CD40 containing NTA_3-DTDA exhibited a 35-fold increase in fluorescence intensity (Fig. 3). The results indicate that even when the concentration of NTA_3-DTDA is 10-fold *lower* than that of NTA-DTDA, the level of PMV binding promoted by NTA_3-DTDA is twice that of NTA-DTDA. PMV modified with POPC, NTA_3-DTDA and FITC-DHPE at molar ratio 96:2:2 (with tracer), or with POPC and NTA_3-DTDA at molar ratio 98:2 (without tracer), therefore, was used for all subsequent experiments outlined below.

3.3. Plasma membrane vesicles engrafted with B7.1 and CD40 target T cells in vivo

The greater ability of NTA_3-DTDA to promote binding of engrafted PMV to cells in vitro led us to explore the effectiveness with which NTA_3-DTDA-modified PMV could target T cells in vivo. Mice were injected into the hind footpad with different liposomal preparations, being namely, conventional liposomes (CL), stealth liposomes (SL) or PMV, all of which contained NTA_3-DTDA, and were engrafted with either EPOR-6H (as control) or B7.1 plus CD40. Draining and non-draining popliteal lymph nodes were removed 14 h after the injection, and lymphocytes isolated from the nodes were incubated with biotinylated CD3ε mAb followed by streptavidin-PE, for analysis by flow cytometry.

Lymphocytes isolated from the draining and non-draining lymph nodes of animals injected with control CL, SL or PMV engrafted with EPOR-6H (not shown), and those isolated from the non-draining lymph nodes of mice injected with CL, SL or PMV engrafted with B7.1-6H and CD40-6H (Fig. 3, panels A–C), all exhibited little or no increase in BODIPY-fluorescence. Lymphocytes isolated from the draining lymph nodes of mice injected with CL engrafted with B7.1-6H and CD40-6H, also showed little, if any, increase in BODIPY-fluorescence (Fig. 4, panel D). In contrast, lymphocytes isolated from the draining lymph node of mice injected with B7.1-6H- and CD40-6H-engrafted SL and PMV exhibited significant increases in BODIPY-fluorescence (Fig. 4, panels E and F). Furthermore, two-colour FACS analysis revealed that lymphocytes exhibiting increased BODIPY-fluorescence also exhibited increased CD3ε mAb binding (Fig. 4, panels H and I). These data

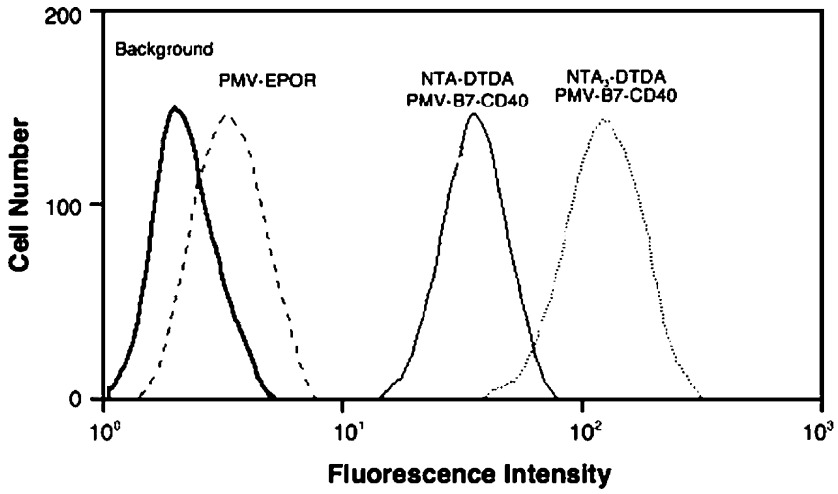

Fig. 3. A comparison of the ability of NTA-DTDA and NTA_3-DTDA to mediate binding of B7.1- and CD40-engrafted PMV to murine D10 cells. Activated D10 cells were incubated (1 h, 4 °C) separately with PMV containing FITC-DHPE as tracer plus either NTA-DTDA (20 mol%) or NTA_3-DTDA (2 mol%), and engrafted with either EPOR (PMV-EPOR) or with B7.1 and CD40 (PMV-B7-CD40), as indicated. The cells were then washed and analysed for FITC-fluorescence by flow cytometry, with the fluorescence profiles reflecting binding of the NTA-DTDA- and NTA_3-DTDA-modified PMV to the cells, as shown. The profile for PMV-EPOR was similar for NTA-DTDA and NTA_3-DTDA. Background fluorescence represents autofluorescence of the T cells. Each fluorescence profile is a representative obtained from three separate experiments performed in duplicate.

indicate that costimulator-engrafted SL and PMV can specifically target T cells in vivo, and that for liposomes, the targeting is critically dependent on the presence of the "stealth" or sterically stabilising lipid PE-PEG_{2000}.

3.4. Cytokines can be incorporated with plasma membrane vesicles

In previous studies, we showed that IL-2 can be incorporated with NTA-DTDA-containing PMV with >95% efficiency [16]. In analogous studies, we assessed the efficiency of incorporation of IL-12, GM-CSF and IFN-γ into NTA_3-DTDA-containing PMV. Cytokine-containing liposomes were prepared and fused with PMV (see Materials and methods), before subjecting the composite PMV to gel filtration, followed by an analysis of the vesicle-incorporated and -unincorporated cytokine fractions by SDS-PAGE (Fig. 5A). Densitometric analysis of the resulting cytokine bands after western transfer and ECL detection of the cytokine bands indicated that under these conditions >85% of the GM-CSF and >75% of the IFN-γ was associated with the membrane vesicle fraction (Fig. 5B). Attempts to quantify encapsulation of IL-12 were unsuccessful due to poor biotinylation and detection of this cytokine by ECL. In the functional studies below, therefore, IL-12 was assumed to be incorporated to a level similar to IFN-γ and GM-CSF.

3.5. B7.1- and CD40-engrafted PMV containing NTA_3-DTDA stimulate T cell proliferation and cytolytic activity

To examine whether engrafted PMV containing NTA_3-DTDA and different cytokines could induce functional effects, B7.1-CD40-engrafted PMV were incubated with splenic T cells for assessment of T cell proliferation in vitro. As depicted in Fig. 6, only low levels of [^{3}H]-thymidine incorporation was observed in control cultures, incorporation being ~4500 cpm for cultures of T cells alone (T cells) and ~6500 cpm for cultures of T cells with PMV engrafted with the EPOR (PMV-EPOR). A higher level of [^{3}H]-thymidine incorporation was observed when T cells were cultured with PMV engrafted with EPOR and containing encapsulated IL-2 (PMV-EPOR-IL-2) (~12,500 cpm), GM-CSF (PMV-EPOR-GM-CSF) (~11,500 cpm), IFN-γ (PMV-EPOR-INF-γ) (~12,600 cpm), IL-12 (PMV-EPOR-IL-12) (~12,700 cpm), and IL-2 and IL-12 (PMV-EPOR-IL-2-IL-12) (~15,000 cpm). The incorporation of [^{3}H]-thymidine was increased to much higher levels, however, in cultures of T cells and PMV bearing engrafted B7.1 and CD40 (PMV-B7-CD40) (~130,000 cpm), PMV bearing engrafted B7.1 and CD40 and containing IL-2 (PMV-B7-CD40-IL-2) (~180,000 cpm), GM-CSF (PMV-B7-CD40-GM-CSF) (~148,000 cpm), IFN-γ (PMV-B7-CD40-IFN-γ) (~157,000 cpm), IL-12 (PMV-B7-CD40-IL-12) (~219,000 cpm), and IL-2 and IL-12 (PMV-B7-CD40-IL-2-IL-12) (~330,000 cpm) (Fig. 6). The results indicate that PMV containing cytokine and engrafted B7.1 and CD40 are considerably more effective than control PMV, in stimulating T cell proliferation.

Engrafted PMV containing NTA_3-DTDA and cytokine also were examined for their ability to stimulate a cytotoxic response in a standard ^{51}Cr release assay. As shown in Fig. 7, cells isolated from control mice immunised either with PBS, or with EPOR-engrafted PMV that either did not contain cytokine (PMV-EPOR) or that contained encapsulated cytokines IL-2 and IL-12 (EPOR-IL-2-IL-12), induced only low levels (4–13%) of lysis at all the effector/target ratios tested (50, 25, 10, 5 and 1). Conversely, compared to T cells from these control mice, the cytolytic activity of T cells isolated from mice immunised with B7.1-CD40-engrafted PMV (PMV-B7-CD40), or with B7.1-CD40-engrafted PMV encapsulating IL-2, IL-12 or IL-2 plus IL-12, was 5-, or 7-, 7.6- and 8.1-fold

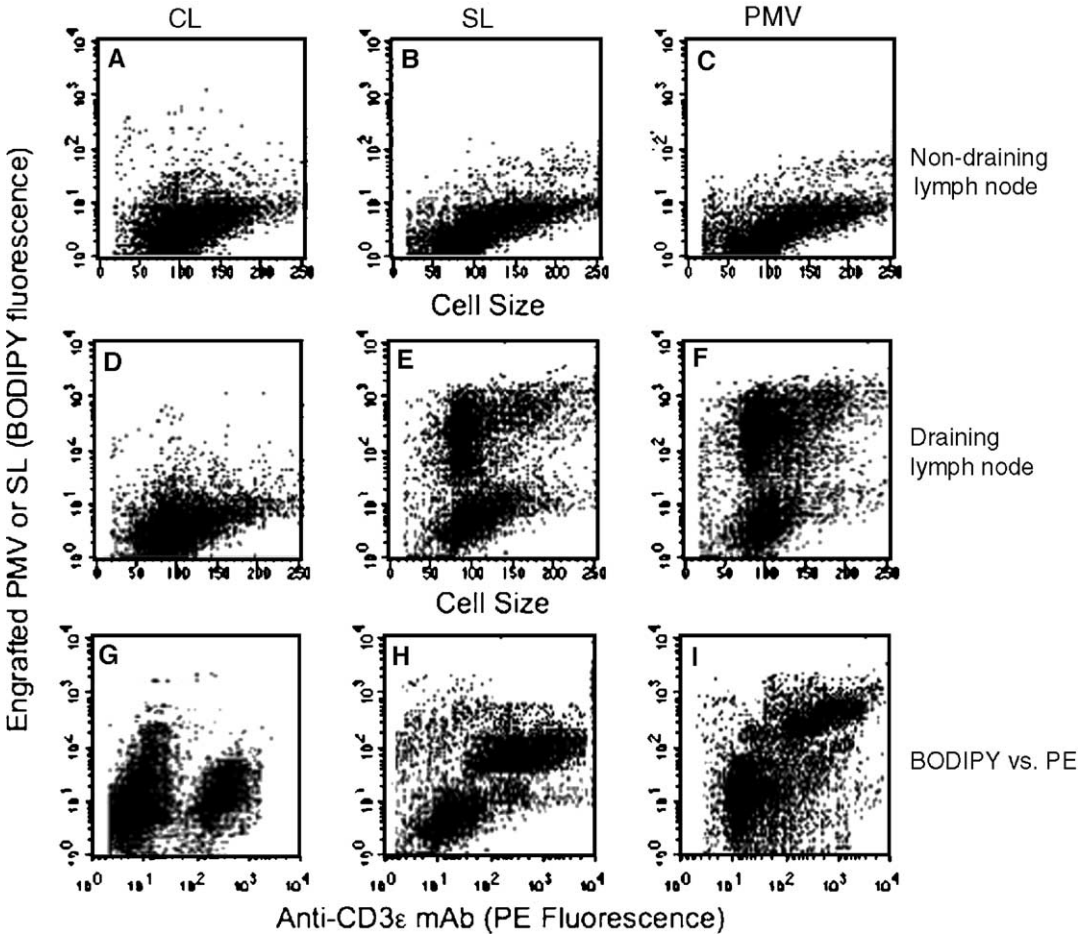

Fig. 4. Targeting of engrafted NTA_3-DTDA-liposomes and PMV to T cells in vivo. NTA_3-DTDA was incorporated into conventional liposomes (CL) lacking PE-PEG_{2000}, stealth liposomes (SL) containing PE-PEG_{2000} or modified PMV (PMV). Each liposome/membrane preparation was then engrafted with either EPOR-6H or B7.1-6H plus CD40-6H, before being injected subcutaneously (hind footpad) into mice. After 14 h, the draining and non-draining popliteal lymph nodes were removed, and lymphocytes isolated and stained with biotinylated-CD3ε mAb followed by streptavidin-PE. The cells were then analysed for BODIPY- and PE-fluorescence by flow cytometry, utilising the intrinsic label (1% BODIPY-PC) of the liposomes and PMV, and staining with streptavidin-PE (to identify $CD3^+$ cells). Panels A–C show dot plots of BODIPY-fluorescence vs. FSC for lymphocytes derived from the non-draining lymph nodes of mice injected with B7.1-C40-engrafted CL, SL or PMV, respectively. Results for draining lymph nodes are shown in panels D–F. The corresponding BODIPY- vs. PE-fluorescence for lymphocytes from draining lymph nodes are shown in panels G–I, respectively. Each plot is representative from two experiments.

higher, respectively, at the effector/target ratio of 50:1 (see Fig. 7). Similarly, the cytolytic activity of T cells for conditions using B7.1-CD40-engrafted PMV encapsulating either GM-CSF or IFN-γ also were tested; for these conditions, the increase in specific lysis induced was ~6-fold above the respective controls (data not shown). In parallel experiments, only background levels of cell lysis were observed when murine A20 cells were used as target cells (not shown), indicating that the observed cytolytic responses are P815 cell-specific.

3.6. Immunisation with engrafted PMV protects against tumor challenge and induces regression of established tumors

To further establish the potential for engrafted PMV as tumor vaccines, DBA/2J mice were immunised with B7.1-CD40-engrafted PMV containing cytokine, and the mice then monitored for tumor growth after challenge with the syngeneic tumor. The data in Fig. 8A show that, relative to mice immunised with PBS or EPOR-engrafted PMV containing IL-2 and IL-12, mice immunised with B7.1-CD40-engrafted PMV exhibited a slower rate of tumor growth, which was more pronounced when the PMV also contained IL-2 and/or IL-12. Thus, 7 weeks after tumor challenge, the mean tumor diameter was 6.3 ± 0.2 mm, 3.3 ± 0.3 mm and 1.0 ± 0.3 mm, for mice immunised with B7.1-CD40-engrafted PMV, B7.1-CD40-engrafted PMV containing either IL-2 or IL-12, and B7.1-CD40-engrafted PMV containing IL-2 plus IL-12, respectively. In contrast, control mice immunised with PBS or with EPOR-engrafted PMV containing IL-2 plus IL-12 had a mean tumor diameter of 13 ± 0.5 mm or 11 ± 0.5 mm, respectively (Fig. 8A). At 12 weeks after tumor challenge, survival ranged from 0 to 15% for mice immunised with PBS or EPOR-engrafted PMV containing cytokines, and 70–85% for mice immunised with B7.1-CD40-engrafted PMV containing cytokines (Fig. 8B). Immunisations with B7.1-CD40-engrafted PMV that contained either GM-CSF or IFN-γ also were carried out, but the anti-tumor effects induced were less pronounced than those observed

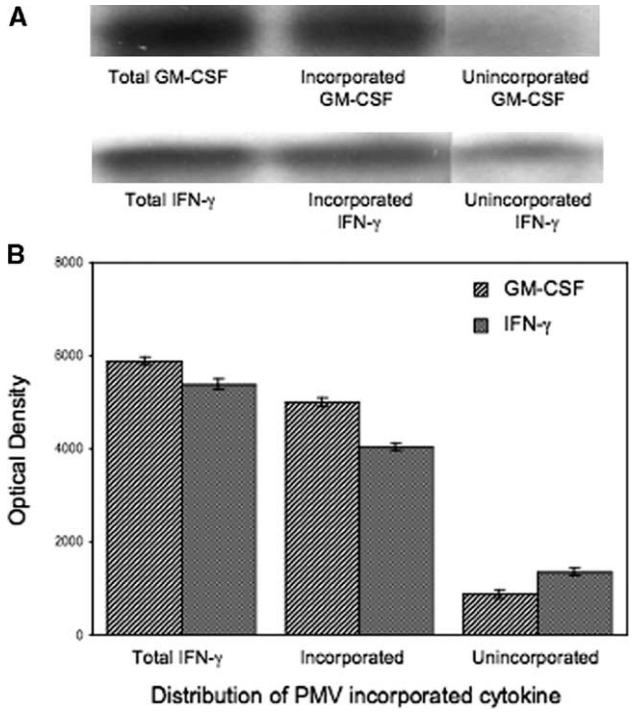

Fig. 5. Engrafted PMV efficiently incorporate cytokines. Synthetic liposomes containing either biotinylated GM-CSF or biotinylated IFN-γ were fused with PMV by sonication and treatment with PEG_{400}. The composite vesicles were purified by size-exclusion chromatography, with the vesicle-incorporated and -unincorporated cytokine fractions being collected separately, and then concentrated. (A) Shows result of ECL detection, with the bands reflecting the cytokine in each fraction after reduced SDS-PAGE analysis, transfer of the proteins onto nitrocellulose membrane, and then probing the membrane with streptavidin-HRP. (B) Shows the relative intensity of the cytokine band representing for each fraction the amount of cytokine relative to the total cytokine used (as indicated). Densitometric analysis was carried out by scanning the film after ECL detection and analysing the intensity of bands using NIH Image 1.61 software.

with IL-2 and/or IL-12 (data not shown). These data indicate that the immunisation of syngeneic mice with engrafted PMV containing cytokines can inhibit tumor growth and prolong survival of the animals after challenge with the native tumor.

To examine whether the engrafted PMV vaccine could induce *regression* of the native tumor, separate groups of DBA/2J mice were challenged by the injection of P815 cells subcutaneously, and the mice then vaccinated three times at 1-week intervals with engrafted PMV containing cytokine, and the mice in each group then monitored for tumor growth and survival. The results show that, compared to control mice immunised with EPOR-engrafted PMV containing cytokine, mice immunised with B7.1-CD40-engrafted PMV containing IL-2 and IL-12, exhibited a significant decrease in the rate of tumor growth, followed by a regression in tumor size (Fig. 9A). Eight weeks after tumor challenge, the mean tumor diameter was 13.0±0.4 mm and 2.0±0.3 mm, for mice immunised with PMV-EPOR-IL-2-IL-12, and PMV-B7-CD40-IL-2-IL-12, respectively (Fig. 9B). At 16 weeks after tumor challenge, survival range was 0–15% for mice immunised with PMV-EPOR-IL-2-IL-12, and 60–75% for mice immunised with PMV-B7-CD40-IL-2-IL-12 (Fig. 9B). These results indicate that the immunisation of syngeneic mice with costimulator-engrafted PMV containing incorporated IL-2 and IL-12 can induce regression of pre-existing P815 tumors in vivo and improve the survival of tumor bearing animals.

4. Discussion

The present work explored the binding properties of the novel chelator lipid NTA_3-DTDA when incorporated into lipid membranes, and the potential for this lipid to be useful for the anchoring of targeting molecules on liposomal membranes for the development of cancer vaccines and/or immunotherapies. The headgroup of NTA_3-DTDA contains three NTA metal chelating moieties, with each NTA being coupled to one of the three COOH groups of a primary NTA-DTDA structure (see Fig. 1). This has produced a lipid in which the hydrophilic headgroup contains three NTA moieties in close proximity, thereby facilitating co-operative interactions with 6His-tagged proteins. In previous studies, utilising the IAsys biosensor, we showed that the interaction of 6H-tagged proteins with NTA-DTDA-containing membranes is significantly more stable when the membrane contains a higher molar ratio of NTA-DTDA to carrier lipid [24]. We envisaged, therefore, that the use of a chelator lipid with a headgroup containing three NTA

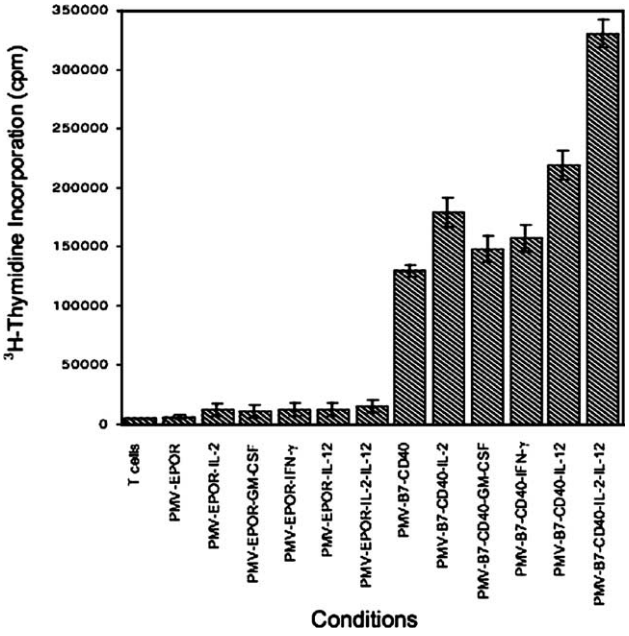

Fig. 6. Stimulation of T cell proliferation by P815 cell-derived PMV containing cytokines and engrafted with costimulatory molecules. Syngeneic DBA/2J splenic T lymphocytes (1×10^5) were incubated alone (T cells), or separately with; control PMV engrafted with EPOR (PMV-EPOR); PMV engrafted with EPOR and containing IL-2 (PMV-EPOR-IL-2), GM-CSF (PMV-EPOR-GM-CSF), IFN-γ (PMV-EPOR-IFN-γ), IL-12 (PMV-EPOR-IL-12) or IL-2 plus IL-12 (PMV-EPOR-IL-2-IL-12); PMV engrafted with B7.1 and CD40 (PMV-B7-CD40); and PMV engrafted with B7.1 and CD40 and containing IL-2 (PMV-B7-CD40-IL-2), GM-CSF (PMV-B7-CD40-GM-CSF), IFN-γ(PMV-B7-CD40-IFN-γ), IL-12 (PMV-B7-CD40-IL-12), or IL-2 plus IL-12 (PMV-B7-CD40-IL-2-IL-12). The cells were cultured for 4 days and then pulsed with 1 μCi of [^{3}H]-thymidine for 16–18 h, before harvesting and measuring radioactivity to assess [^{3}H]-thymidine incorporation. Results are shown as counts per minute (cpm)±S.E.M.

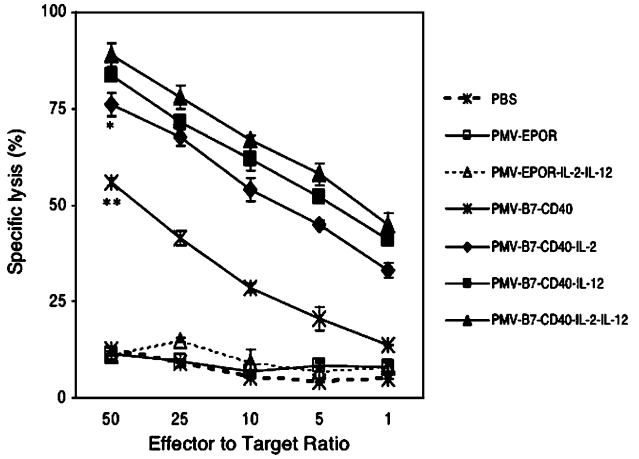

Fig. 7. Induction of tumor-specific cytotoxic responses in mice immunised with costimulator-engrafted PMV containing cytokines. Mice were immunised subcutaneously with PBS, or with: P815 cell-derived PMV engrafted with EPOR (PMV-EPOR); PMV engrafted with EPOR and containing IL-2 plus IL-12 (PMV-EPOR-IL-2-IL-12); PMV engrafted with B7.1 and CD40 (PMV-B7-CD40); and PMV engrafted with B7.1 and CD40 and containing IL-2 (PMV-B7-CD40-IL-2), IL-12 (PMV-B7-CD40-IL-12), or IL-2 plus IL-12 (PMV-B7-CD40-IL-2-IL-12). After 14 days splenic T cells were isolated and co-cultured with γ-irradiated native P815 cells for 5 days before being incubated for a further 6 h with ^{51}Cr-labelled native P815 cell targets (at the indicated E:T ratio) for determination of specific lysis in a standard ^{51}Cr release assay. Results are expressed as the percentage specific lysis ± S.E.M. At 50:1 E:T ratio, *indicates specific lysis is significantly higher: ($n=6$, $^{***}P<0.001$) than control mice immunised with either PBS or EPOR-engrafted PMV (with or without cytokine); ($n=6$, $^{**}P<0.01$) than mice immunised with B7.1-CD40-engrafted PMV not containing cytokine.

moieties (i.e., as in NTA_3-DTDA), instead of one (as in NTA-DTDA) would have advantages in applications for the study of molecular interactions on lipid membranes, as well as for the development of vaccines and/or targeted liposomal agents.

An analysis of the binding of B7.1-6H to membranes containing either NTA-DTDA or NTA_3-DTDA, with the IAsys biosensor revealed that B7.1-6H interacts more stably with membranes containing NTA_3-DTDA. For these studies a 3-fold higher concentration of NTA-DTDA compared to NTA_3-DTDA was used so as to compare binding to the same total number of NTA moieties on the deposited membranes. The results show that the on-rate for binding of B7.1-6His was faster for NTA-DTDA-membranes ($2.62\pm0.06\times10^4$ M^{-1} s^{-1}), than for NTA_3-DTDA-membranes ($1.55\pm0.08\times10^4$ M^{-1} s^{-1}). However, binding to NTA_3-DTDA-membranes generally exhibited a slower off-rate (i.e., *fast* and *slow* components) and a 1- to 3-fold lower $K_{d,}$ at all of the B7.1-6His concentrations tested (see Fig. 2 and Table 1). The results demonstrate that, even when the total number of NTA moieties on the membrane is the same, the binding of His-tagged proteins to NTA_3-DTDA-membranes is markedly more stable than that to NTA-DTDA-membranes.

A detailed analysis of the nature of the interaction of 6H-tagged proteins with NTA_3-DTDA, was considered beyond the scope of the present work. However, each NTA moiety can bind one divalent metal ion (Ni^{2+}), and each Ni-NTA can, in turn, interact with two successive histidine residues. The greater stability of 6H-tagged proteins anchored onto NTA_3-DTDA-membranes, therefore, is likely to be due to a higher local density of Ni-NTA moieties, compared to NTA-DTDA-membranes. Since each molecule of NTA_3-DTDA has three Ni-NTA groups, all six histidine residues can potentially interact simultaneously with one Ni-NTA_3-DTDA molecule, facilitating co-operative interactions and stable binding of the 6H-tagged protein. Each Ni-NTA-DTDA, on the other hand, can simultaneously interact with only two successive His residues, thereby enabling stable interactions with 6H-tagged proteins to occur only at high NTA-DTDA concentrations.

Previously, we showed that NTA-DTDA can be incorporated into liposomes and PMV for engraftment of 6H-tagged proteins for liposome targeting and vaccine development [16]. To determine whether NTA_3-DTDA also can be used to engraft

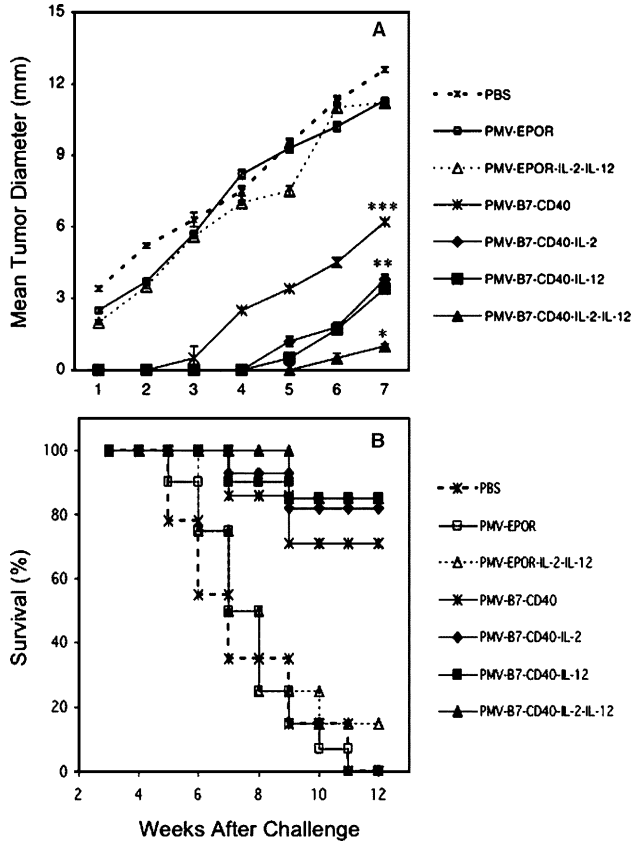

Fig. 8. Anti-tumor responses in syngeneic mice immunised with costimulator-engrafted PMV containing cytokines. Separate groups of mice (7 mice in each group) were immunised subcutaneously with PBS, or with: P815 cell-derived PMV engrafted with EPOR (PMV-EPOR); PMV engrafted with EPOR and containing IL-2 plus IL-12 (PMV-EPOR-IL-2-IL-12); PMV engrafted with B7.1 and CD40 (PMV-B7-CD40); and PMV engrafted with B7.1 and CD40 and containing IL-2 (PMV-B7-CD40-IL-2), IL-12 (PMV-B7-CD40-IL-12), or IL-2 plus IL-12 (PMV-B7-CD40-IL-2-IL-12), as indicated. Two weeks after vaccination the mice in each group were challenged by subcutaneous injection of native P815 cells (1×10^5), and the mice then monitored for tumor growth. Each point in panel A represents the mean tumor diameter for each group of mice as a function of time for the first 7 weeks. At week 7, *indicates significance of tumor growth: ($n=6$, $^{***}P<0.001$) with respect to control mice (immunised with either PBS, or with EPOR-engrafted PMV with or without cytokine); ($n=6$, $^{**}P<0.01$) with respect to mice immunised with B7.1-CD40-engrafted PMV not containing cytokine; ($n=6$, $^{*}P<0.05$) with respect to B7.1-CD40-engrafted PMV containing either IL-2 or IL-12. The data in panel B show the percentage survival of the animals with time.

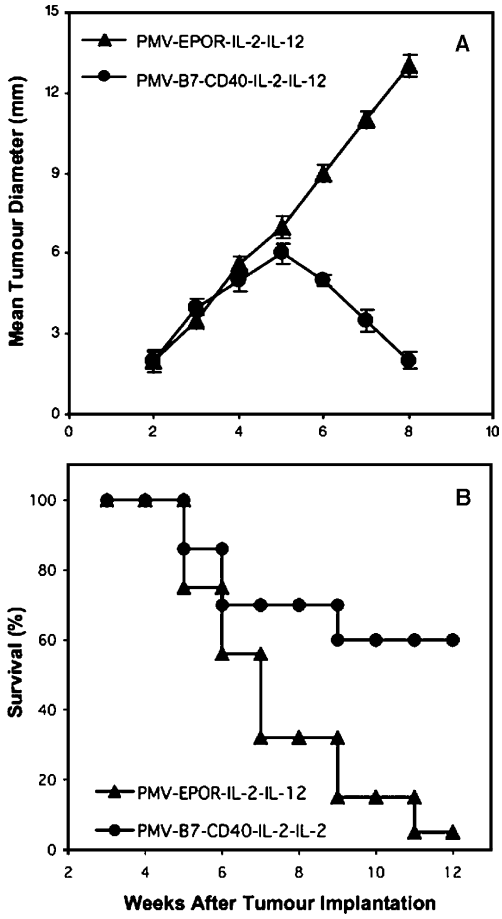

Fig. 9. Immunisation of syngeneic mice with costimulator-engrafted PMV containing IL-2 and IL-12 induces regression of established tumors. Separate groups of mice were injected subcutaneously with 1×10^5 native P815 cells. Two weeks after injection the mice were immunised subcutaneously with either P815 cell-derived control PMV engrafted with EPOR containing IL-2 plus IL-12 (PMV-EPOR-IL-2-IL-12); or PMV engrafted with B7.1 and CD40 and containing IL-2 and IL-12 (PMV-B7-CD40-IL-2-IL-12) as indicated. The mice in each group were then monitored for tumor growth. The data in panel A show the mean tumor diameter of each group of mice as a function of time for the first 8 weeks. The data in panel B show the percentage survival of the animals with time.

targeting proteins onto liposomal membranes, we tested the ability of liposomal membranes containing NTA_3-DTDA and engrafted with B7.1 and CD40, to interact specifically with cells. For these and subsequent functional studies, we used concentrations (molar ratios) of NTA_3-DTDA that were 10-fold lower than those used for NTA-DTDA. In vitro studies in which B7.1-CD40-engrafted PMV derived from P815 tumor cells were incubated with D10 cells in vitro showed that the engrafted PMV bound T cells in a receptor–ligand-specific manner (Fig. 3). Importantly, the administration of B7.1-CD40-engrafted PMV and stealth liposomes to mice by hind footpad injection resulted in an increased number of labelled T cells being isolated in the draining popliteal lymph node, indicating that these can target T cells in vivo. Interestingly, no targeting was observed with conventional liposomes that lack PE-PEG_{2000} (Fig. 4), suggesting that the inclusion of this "stealth" lipid is crucial for effective targeting of liposomes in vivo, presumably by reducing non-specific binding to other blood components.

Consistent with the observed stronger interaction of B7.1-6H with NTA_3-DTDA-containing membranes (Fig. 2, Table 1), the results show that B7.1-CD40-engrafted liposomal membranes containing NTA_3-DTDA also are very effective at mediating specific binding to T cells in vitro and in vivo (Figs. 3 and 4). Importantly, B7.1 and CD40 once engrafted onto PMV via incorporated NTA_3-DTDA, also could be shown to induce functional effects in assays of syngeneic T cell proliferation and cytolytic activity. The large increases in T cell proliferation observed (Fig. 6) suggests that in these studies a substantial component of the proliferation induced is due to the mitogenic effect of cross-linking the CD28 molecule on T cells by liposome-engrafted CD80. However, since vaccination of animals with CD80-bearing PMV containing IL-2 and IL-12, *does not* induce significant specific cytolytic responses or offer protection against growth of the P815 tumor (or provide survival advantage to mice) when the PMVs used in the immunisation are derived from the EL-4 lymphoma instead of P815 cells (not shown), the results demonstrate that the anti-tumor effect of the vaccine is antigen-specific.

Considerable evidence suggests that the stimulatory capacity of a vaccine may depend on the presence of cytokines. In the present work, cytokines were encapsulated into NTA_3-DTDA-containing PMV engrafted with costimulatory molecules, thus permitting the simultaneous delivery of antigen, costimulatory signals and cytokine to T cells. For encapsulation into PMV, cytokines were first incorporated into synthetic liposomes containing NTA_3-DTDA, and the liposomes then fused with P815-derived PMV. Our results show that encapsulation of IFN-γ and GM-CSF into PMV in this way occurs with high efficiency (>75%–85%, Fig. 5), similar to that of IL-2 [16]; significant incorporation of IL-12 also could be demonstrated by functional studies (Figs. 7 and 8). While IL-2, IL-12, GM-CSF and IFN-γ all have been purported to exhibit beneficial effects in the treatment of tumors [12,18,19], in the present work strong anti-tumor responses were induced by costimulator-engrafted PMV containing IL-2 and/or IL-12, with those containing GM-CSF and IFN-γ inducing a smaller effect. Interestingly, costimulator-engrafted PMV containing both IL-2 and IL-12 induced additive effects on T cell proliferation (Fig. 6), and inhibited tumor growth and prolonged survival of syngeneic mice challenged with the native P815 tumor (Fig. 8). Importantly, the immunisation of tumor-bearing mice with B7.1-CD40-engrafted PMV containing IL-2 and IL-12 could induced regression of established tumors and prolong survival of these animals (Fig. 9). These observations are consistent with an ability of B7.1-CD40-engrafted PMV to interact specifically with T cells (Figs. 3 and 4), and with the reported ability of IL-2 and IL-12 to stimulate T cell activation and cytolytic activity in vivo [11].

A major finding from the present work is that the novel lipid NTA_3-DTDA can be used to anchor 6H-tagged proteins with greater stability and effectiveness than NTA-DTDA. Thus, while achieving responses which are directly comparable and essentially the same as those previously obtained with NTA-DTDA (Ref. [16] see Figs. 6 and 7), the present work shows that the induction of tumor-specific cytotoxic responses (Fig.

7), the inhibition of tumor growth (Fig. 8A), and the effect on prolongation of survival (Fig. 8B), can all be achieved at concentrations of NTA_3-DTDA that are 10-fold *lower* than those previously required for NTA-DTDA [16]. These results are consistent with the binding data presented in Figs. 1–3 and Table 1, which show that the stability of membrane-anchored CD80 is much greater for NTA_3-DTDA than for NTA-DTDA—even when the NTA-DTDA is at a 3-fold *higher* concentration. The findings thus demonstrate that NTA_3-DTDA is potentially a more useful lipid for both the study of molecular interactions on model membranes, and the development of targeted membranes and liposomal agents for the development of tumor vaccines and immunotherapies. Consistent with our previous findings with NTA-DTDA [16], tumor cell-derived PMV containing NTA_3-DTDA, allow engraftment of costimulatory molecules onto the surface of the PMV, and encapsulation of cytokine, for simultaneous delivery of PMV-associated antigens, costimulatory signals and cytokines. Thus, effective responses against the P815 tumor can be generated in mice immunised with PMV containing encapsulated cytokines and bearing engrafted costimulatory molecules. In the present work, we also show that the vaccination of animals subsequent to their inoculation with the P815 tumor can induce a slowing of the rate of growth, followed by a regression of tumor growth, prolonging survival of the animals (Fig. 9). The results show that vesicle-engrafted B7.1 and CD40 coupled via NTA_3-DTDA also are functionally active, that the encapsulated cytokines can provide concomitant stimulation, and that antigens on the modified PMV can still stimulate anti-tumor responses in a syngeneic system.

The modified PMV described herein offer several distinct advantages over other targeted liposome systems, or the use of immunostimulatory-gene transfected tumor cells in the development of anti-tumor vaccines. Firstly, PMV may be prepared from tumor tissue and stored frozen until required, avoiding the need to establish primary cultures of tumor cells for transfection [28]. Secondly, modification of the PMV can be achieved in a relatively short period of time (~2 h), unlike the genetic transfection of cells [29,30]. The modified PMV can be engrafted with many different His-tagged proteins simultaneously, and encapsulate a number of different cytokine(s), whereas multiple transfections and expression of different molecules simultaneously can be difficult to achieve in practice [31]. The results show that tumor-derived PMV modified in this way can target T cells in vivo, stimulating specific anti-tumor immune responses and regression of established tumors in the P815 model. It is envisaged that the system described could be adapted to target a number of agents to various cells in vivo, as for example the delivery of antigens to dendritic cells for the induction or suppression of immune responses [32].

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