Targeting Dendritic Cells with Antigen-Containing Liposomes: A Highly Effective Procedure for Induction of Antitumor Immunity and for Tumor Immunotherapy

Christina L. van Broekhoven,¹ Christopher R. Parish,² Caroline Demangel,³ Warwick J. Britton,⁴,⁵ and Joseph G. Altin¹

¹School of Biochemistry and Molecular Biology, Faculty of Science, ²Division of Immunology and Genetics, John Curtin School of Medical Research, The Australian National University, Canberra, Australian Capital Territory, Australia; ³Institut Pasteur, Unité de Génétique Moléculaire Bactérienne, Paris, France; and ⁴The Centenary Institute of Cancer Medicine and Cell Biology and ⁵Discipline of Medicine, University of Sydney, Sydney, New South Wales, Australia

ABSTRACT

Dendritic cells (DCs) are potent stimulators of immunity, and DCs pulsed with tumor antigen ex vivo have applications in tumor immunotherapy. However, DCs are a small population of cells, and their isolation and pulsing with antigen can be impractical. Here we show that a crude preparation of plasma membrane vesicles (PMV) from the highly metastatic murine melanoma (B16-OVA) and a surrogate tumor antigen (OVA) can be targeted directly to DCs in vivo to elicit functional effects. A novel metal-chelating lipid, 3(nitrilotriacetic acid)-ditetradecylamine, was incorporated into B16-OVA-derived PMV, allowing recombinant hexahistidine-tagged forms of single chain antibody fragments to the DC surface molecules CD11c and DEC-205, to be conveniently “engrafted” onto the vesicle surface by metal-chelating linkage. The modified PMV, or similarly engrafted synthetic stealth liposomes containing OVA or OVA peptide antigen, were found to target DCs in vitro and in vivo, in experiments using flow cytometry and fluorescence confocal microscopy. When used as vaccines in syngeneic mice, the preparations stimulated strong immune responses having hitherto focused primarily on procedures involving the manipulation of DCs ex vivo. This approach often requires that blood DCs be isolated from a patient, the cells then exposed to antigen and matured in culture, and then reintroduced into the patient (2–5). Whereas this procedure is simple in principle, there are difficulties associated with the isolation of such minor cell populations, which are present in relatively low numbers in blood (6, 7). Clearly, strategies that deliver antigens directly to DCs in vivo and that can elicit an appropriate immune response have enormous clinical potential.

DCs originate from progenitors in the bone marrow and migrate as immature cells to peripheral tissues where they internalize antigen and undergo a complex maturation process. Antigen is internalized via a number of surface receptors, including the complement receptor CD11c/CD18 (8–10) and the endocytic receptor DEC-205 (11, 12). During antigen acquisition, immature DCs also may receive “danger” signals in the form of pathogen-related molecules such as bacterial cell wall lipopolysaccharide (LPS), or maturation and/or inflammatory stimuli via cytokines such as IFN-γ. DCs then migrate to the secondary lymphoid organs, maturing to become competent antigen presenting cells (13). The receptors CD11c/CD18 and DEC-205 are believed to play a crucial role in the process of antigen capture and presentation and are expressed almost exclusively on DCs. It is conceivable, therefore, that both receptors also could be used for targeting antigen directly to DCs in vivo. Consistent with this notion, a fusion protein consisting of antigen fused with antibodies (Ab) to DEC-205, and a DEC-205 monoclonal antibody (mAb) chemically conjugated with antigen have been shown to target DCs in vivo, inducing T-cell activation when coadministered with inflammatory stimulators such as anti-CD40 Ab (14, 15).

Synthetic liposomes have the potential to deliver large quantities of antigen to DCs (16–18), but to date their targeting to specific surface molecules on DCs has been difficult to achieve in practice (18–23). Clearly, an effective strategy that combines the antigen carrying capacity of liposomes and the specificity of molecular recognition to target multiple antigens and maturation and/or “danger” signals directly to DCs in vivo would have enormous potential in simplifying DC immunotherapies. Here we examine whether a novel chelator-lipid, 3(nitrilotriacetic acid)-ditetradecylamine (NTA₃-DTDA) can be used to anchor histidine-tagged forms of single chain full-length variable Ab fragments (ScFv), which target DCs, onto either tumor-derived plasma membrane vesicles (PMV) or onto antigen-containing stealth liposomes. The results show that the targeting of antigen to DCs in this way is highly effective at inducing immunity and protection against tumor, with protection being at least partially dependent on the eosinophil chemokine eotaxin.

INTRODUCTION

Dendritic cells (DCs) are a small population of antigen presenting cells uniquely capable of stimulating primary immune responses, and a strong interest has developed in their use in cancer immunotherapies (1). Attempts to harness the capacity of DCs to stimulate potent immune responses have hitherto focused primarily on procedures involving the manipulation of DCs ex vivo. This approach often requires that blood DCs be isolated from a patient, the cells then exposed to antigen and matured in culture, and then reintroduced into the patient (2–5). Whereas this procedure is simple in principle, there are difficulties associated with the isolation and culture of such minor cell populations, which are present in relatively low numbers in blood (6, 7). Clearly, strategies that deliver antigens directly to DCs in vivo

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Requests for reprints: Joseph G. Altin, School of Biochemistry and Molecular Biology, Faculty of Science, The Australian National University, Canberra Australian Capital Territory 0200, Australia. Phone: 61-2-6125-4495; Fax: 61-2-6125-0313; E-mail: Joseph.Altin@anu.edu.au

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Monoclonal Abs and Proteins

Murine CD56 (clone 42.18; rat IgG2a) mAb was from the 6th Human NK Cell Workshop and the murine CD3 mAb (clone 145–2C11; Armenian hamster IgG) was purchased from PharMingen (San Diego, CA). Recombinant murine IFN-γ and granulocyte macrophage colony-stimulating factor (GM-CSF) were supplied by PeproTech Inc. (Rocky Hill, NJ). Recombinant ScFv Abs N418 (anti-CD11c) and NLDC145 (anti-DEC-205; Refs. 25, 26) were developed employing the VH and VL regions amplified from cDNA from the respective hybridomas and shown to bind to murine DCS. A hexahistidine (6H) tag was incorporated at the COOH terminal of the Abs, CD11c-ScFv and DEC-205-ScFv, respectively, and these were expressed in the baculovirus expression system and purified as described previously (27). Peptides were synthesized by the Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University, Canberra. The L2 peptide (GHHHPHHGHHPH), a sequence of 10 amino acids found in the plasma protein histidine-rich glycoprotein, was used routinely to engraft control PMV and stealth liposome (SL) because it binds to Ni-NTA-DTDA with high avidity and can block its nonspecific binding to cells. The peptide SIINFEKL-6H, representing the immunodominant CTL epitope of OVA in H-2 b mice (OVA), and can block its nonspecific binding to cells. The peptide SIINFEKL-6H, representing the immunodominant CTL epitope of OVA in H-2 b mice (OVA), and can block its nonspecific binding to cells. The peptide SIINFEKL-6H, representing the immunodominant CTL epitope of OVA in H-2 b mice (OVA), and can block its nonspecific binding to cells. The peptide SIINFEKL-6H, representing the immunodominant CTL epitope of OVA in H-2 b mice (OVA), and can block its nonspecific binding to cells.

Mice and Cell Lines

Female or male C57BL/6 mice (H-2 b) 6–8 weeks of age were supplied by the Animal Breeding Establishment, and C57BL/6 cd8a knock out mice (H-2 b, eotin/x-) were obtained from Dr. Paul Foster, Division of Molecular Bioscience, John Curtin School of Medical Research (Australian National University). All of the animal experimentation protocols were approved by the Australian National University Animal Experimentation Ethics Committee. The highly metastatic murine B16-OVA melanoma (C57BL/6 (H-2 b)), an OVA-secreting tumor cell line, was cultured at 37°C in an atmosphere of 5% CO2 in RPMI 1640 (Life Technologies, Inc., Invitrogen, Melbourne, Australia) containing 10% FCS (Trace Biosciences, Noble Park, Victoria, Australia) and 0.5 mg/ml Geneticin (Invitrogen). Murine fetal skin DCs (FSDC) (C57BL/6-DOM/4, H-2 b) were cultured in the same medium but without geneticin. Murine long-term culture DCs (LTC-DC) (B10.A(2R; H-2 k/b)) isolated and cultured as described (28), were a gift from Prof. Helen O’Neill (School of Biochemistry and Molecular Biology, Australian National University).

Isolation of T Cells

Murine T cells were isolated from the spleens of C57BL/6 mice. The spleens were dissociated into single cell suspensions, and after removing red cells by hypotonic lysis, the T cells were isolated using a nylon wool column (29). Although this procedure leads to an enrichment in the number of T cells, it does not substantially deplete the number of DCs in the purified cell population. Hence, it can be expected that antigen presentation and cross-presentation could still occur in lymphocyte cultures with these cells.

Engrafted PMVs and SLs

PMVs. PMV from cultured cells were prepared by sucrose gradient centrifugation as described previously (30, 31). Briefly, cultured B16-OVA cells (1 × 108) were washed twice with PBS to remove proteins from the culture medium. The cells were suspended in homogenization buffer [10 mM Na2HPO4/NaH2PO4 (pH 7.4) containing 30 mM NaCl, 1 mM MgCl2, and 0.02% NaN3] and homogenized by brief sonication at 4°C. The cell lysate was then layered over a 41% sucrose gradient and centrifuged (95,000 × g for 1 h at 4°C). The PMVs were collected from the interfacial band and washed twice in homogenization buffer by centrifugation (95,000 × g; 20 min; 4°C). Different batches of PMVs were always prepared from a known starting number of cells after the same procedure; each batch of PMVs was standardized for total protein and number of “cell equivalents,” with typically 1 × 108 cell equivalents of PMVs being suspended in 100 μl of PBS. Stock PMV suspensions were stored at −20°C and were briefly resorbed before use in each experiment.

Modification of PMVs. A procedure similar to that used for the incorporation of NTA-DTDA into PMVs (31) was used also for the incorporation of NTA-DTDA into PMV, because this method was found to be optimal when assessed in studies (data not shown) of the binding of engrafted PMVs to cells in flow cytometry experiments analogous to those shown in Fig. 2A. For the present work the liposomes used to modify PMVs were prepared as follows: ethanolic solutions of POPC, NTA2-DTDA, LPS, and PC-BODIPY (molar ratio 94:2:2:2) or POPC, NTA2-DTDA, and PC-BODIPY (molar ratio 96:2:2), were mixed, dried under a stream of N2, then rehydrated in 100 μl of PBS containing 60 μM Ni2+. Where indicated, as an alternative to LPS, either IFN-γ or GM-CSF (50 ng) were included in the rehydration buffer. Hydrated mixtures were sonicated (three times; 15 s bursts on ice) using a TOSCO 100W ultrasonic disintegrator (Measuring and Scientific Ltd., London, United Kingdom) at maximum amplitude. In initial studies the efficiency of LPS/cytokine incorporation in liposomes was assessed after purification by gel filtration and analysis of the “free” and liposome-associated LPS/cytokine by SDS-PAGE; in all of the instances >75% incorporation was obtained (data not shown). Furthermore, consistent with previous reports, our studies showed that liposomes with incorporated LPS bound to FSDC, and liposomes with incorporated IFN-γ and GM-CSF were functionally active, with GM-CSF-containing liposomes stimulating proliferation when added to cultures of FSDC in serum-free medium and IFN-γ-containing liposomes inhibiting FSDC proliferation in serum-containing medium (data not shown).

Liposomes prepared in this way (with or without incorporated LPS or cytokine) were then used to modify PMVs. Briefly, 100 μl of the liposome suspension was mixed with 100 μl of B16-OVA cell-derived PMVs (1 × 108 cell equivalents) before adding 15% PEG400 and diluting 10 times with PBS. The NTA2-DTDA- and cytokine-containing PMVs were then purified by size-exclusion chromatography (31) before engrafting with T-cells (see below). SLs. SLs were prepared as follows: POPC, NTA2-DTDA, PE-PGEO500, LPS, and PC-BODIPY (molar ratio 96:1:1:1:1); or POPC, NTA2-DTDA, PE-PGEO500, and PC-BODIPY (molar ratio 97:1:1:1) dissolved in ethanol were dried under a stream of N2 then rehydrated in 100 μl PBS containing 30 μM Ni2+ (total lipid 1 mm). Preliminary studies showed that the inclusion of 1% PE-PGEO500 in the SL lipid mixture had no significant effect on the efficiency of engraftment of either of the two hexahistidine-tagged ScFvs to be used for the targeting of the engrafted SL to LTC-DC in vitro (data not shown), when assessed in binding studies by flow cytometry. For mixtures lacking LPS, IFN-γ or GM-CSF (50 ng) was included in the PBS (as described for modification of PMV; see above). Lipid mixtures were sonicated and SL purified (as above). For functional studies the PC-BODIPY was omitted from all of the lipid mixtures.

Incorporation of OVA and SIINFEKL Peptide in SLs. Encapsulation of the immunodominant epitope of the OVA protein, SIINFEKL, into SL was attempted but proved difficult because this peptide has low solubility at the pH used to produce the SL and to engraft histidine-tagged ScFv (pH 7.4). However, a hexahistidine-tagged form of the peptide, SIINFEKL-6H, permitted efficient encapsulation and/or engraftment of the peptide onto NTA2-DTDA-containing SL. Binding studies using fluorescence-activated cell sorter analysis showed that CD11c-ScFv- or DEC-205-ScFv-engrafted SL containing SIINFEKL-6H could effectively target receptors on DCs in vitro, provided that the amount of SIINFEKL used did not exceed 2 μM (data not shown). Thus, where indicated, SIINFEKL-6H (2 μM) was included to simultaneously engraft ScFv and SIINFEKL-6H, by the efficient encapsulation of OVA into SL containing POPC, NTA2-DTDA, and PE-PGEO500, was achieved by rehydrating the desiccated lipid mixture in PBS containing 0.1 mg of OVA (1 mg/ml) followed by brief sonication. In preliminary studies the total amount of OVA that was encapsulated was checked by SDS-PAGE analysis of the total amount of OVA in the “free” and SL-associated fractions after separation by gel filtration. Under the conditions used the efficiency of OVA encapsulation was >85%. OVA- or SIINFEKL-containing SLs were always prepared from known amounts of proteins, OVA, and SIINFEKL peptide, and an unassociated material was removed from the SL or modified PMV preparation by gel filtration before engraftment of ScFv for targeting and use in experiments.

Engraftment of ScFv onto Modified PMV and SL. The engraftment of targeting proteins onto the modified PMV and SL was carried out by a procedure similar to the engraftment of T-cell costimulatory molecules (31).

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Thus, hexahistidine-tagged ScFv to murine CD11c or DEC-205, dissolved in PBS, was incubated with a suspension of the SL or PMV (that had been modified to contain the NTA3-DTDA lipid and the appropriate incorporated LPS, cytokine, and/or the OVA antigen) for 1 h at room temperature. The amount of ScFv used in the engrafment was in the same molar ratio as the effective concentration of NTA3-DTDA used for preparing the SL or modified PMV preparations. This concentration was found to give optimal binding of the ScFv-engrafted SLs and PMV to LTC-DC and FS-DC in preliminary experiments using flow cytometry (data not shown), in studies analogous to those presented in Fig. 2A.

Targeting of DC in Vivo

For tracking studies, fluorescent PMV were obtained by reacting the PMV with FITC (Molecular Probes); they were then engrafted with L2 or ScFv, and injected into the hind footpad of mice. After 16 h the draining popliteal lymph node of each animal was harvested and used either for isolation of lymph node cells for two color flow cytometric analysis after staining with biotinylated CD11c mAb and streptavidin-phycocerythrin or for confocal fluorescence imaging. For imaging, lymph nodes were fixed in 10% formalin, then embedded in paraffin, and cut into sections; the sections were then adhered onto slides and dewaxed. Slides were blocked by incubation with PBS plus 20% goat serum for 30 min at room temperature, before incubating with mAb N418 to CD11c in PBS plus 20% goat serum, and then stained with streptavidin-phycocerythrin in PBS plus 20% goat serum. Additional washing, the slides were analyzed for fluorescein and rhodamine fluorescence using a Radiance 2000 confocal fluorescence confocal microscope (Bio-Rad, Richmond, CA). Images were acquired by Kalman averaging of 30 successive laser scans and processed using Bio-Rad Image software.

Cytotoxicity Assays

Antigen-specific CTL assays were performed similar to those described (32). Syngeneic C57BL/6 mice were immunized i.v. with PBS (control) or ScFv-engrafted B16-OVA cell-derived PMV or SL bearing antigen (as indicated). At day 14 after immunization, spleens were removed, and T lymphocytes (effector T cells) were isolated as above. The T cells were then suspended in complete growth medium and aliquoted into 24-well flat-bottomed plates (ICN Biomedicals) at a concentration of 1 x 10^6 cells/well and cocultured with 1 x 10^5 γ-irradiated (5000 rad) B16-OVA cells. After 5 days of coculture, the cytolytic activity of the T cells was assessed in a standard 3HCr release assay, as described (31).

Immunization of Animals and Tumor Challenge in Vivo

Mice were immunized by three i.v. injections given weekly, with PBS (control) or either ScFv-engrafted B16-OVA cell-derived PMV or SL bearing antigen (as indicated). At day 14 after immunization, spleens were removed, and T lymphocytes (effector T cells) were isolated as above. The T cells were then suspended in complete growth medium and aliquoted into 24-well flat-bottomed plates (ICN Biomedicals) at a concentration of 1 x 10^6 cells/well and cocultured with 1 x 10^5 γ-irradiated (5000 rad) B16-OVA cells. After 5 days of coculture, the cytolytic activity of the T cells was assessed in a standard 3HCr release assay, as described (31).

RESULTS

Liposomes Can Be Used to Target Antigens to DCs Both in Vitro and in Vivo. Two types of liposome preparations were used as vectors to target DCs (Table 1). The first entailed the use of a crude preparation of tumor cell-derived PMV modified by engrafment of ScFv targeting DCs, and the second was a preparation of antigen-containing SLs also engrafted with DC targeting ScFv. SLs, also known as stericly stabilized liposomes, are synthetic lipid structures, which have been sterically stabilized by the inclusion of lipids such as PE-PEG3000, and, by virtue of their ability to escape nonspecific elimination by the reticuloendothelial system, can remain in the blood circulation for days after their i.v. administration (18, 20, 33). The use of the chelator lipid NTA3-DTDA to modify tumor cells and tumor cell-derived PMV for engrafment of T-cell costimulatory molecules has been described (31, 34). We have produced recently a novel lipid, NTA3-DTDA (Fig. 1A), which is related to NTA-DTDA, but by achieving a higher local density of NTA head groups, can permit a more stable anchoring (effective Δ5–10-fold lower for NTA3-DTDA than for NTA-DTDA) of histidine-tagged proteins onto NTA3-DTDA-containing SLs and modified PMV. Thus, liposome attachment, via NTA3-DTDA, of histidine-tagged ScFv against DC markers such as CD11c and DEC-205 should allow effective targeting of the SLs (Fig. 1B) and modified PMV (Fig. 1C) to DCs.

To determine whether the two types of liposomes can be used as vectors to target DCs, we first explored the ability of this system to target DCs in vitro. In this study we used the highly metastatic melanoma cell line, B16-OVA, as this line secretes low levels of OVA, which can be used as a surrogate secreted tumor-specific antigen (35), enabling OVA-specific immune responses to be assessed. The B16-OVA tumor line is largely resistant to OVA-specific CTLs unless high avidity CTLs are used (35). PMV (B16-OVA-derived) were modified to contain an engrafted targeting protein, namely the L2 peptide (used as control) or a ScFv to either CD11c or DEC-205 (see “Materials and Methods”). Similarly, engrafted SL containing OVA or the OVA CTL epitope SIINFEKL were produced (see Table 1). A summary of the different liposome and modified PMV preparations used in this study is shown in Table 1. Because the modified membranes also were made to contain PC-BODIPY as a fluorescent tracer, their targeting to DCs could be assessed by flow cytometry.

LTC-DCs exhibited little binding of control-modified PMV (PMV-L2; ~2-fold increase in fluorescence above background), but when incubated with PMV engrafted with either CD11c-ScFv (PMV-CD11c) or DEC-205-ScFv (PMV-DEC-205), there was a 4–8-fold increase in binding above control cells (Fig. 2A). A similar result was obtained when LTC-DCs were incubated with SL engrafted with CD11c-ScFv (SL-CD11c) and DEC-205-ScFv (SL-DEC-205; data not shown). Similarly, the incubation of FS-DC expressing CD11c, with PMV or SLs engrafted with CD11c-ScFv, resulted in a fluorescence increase substantially above that of control cells (data not shown). Importantly, preincubation of LTC-DCs with either the anti-
TARGETING ANTIGEN TO DENDRITIC CELLS IN VIVO

To establish whether the ScFv-engrafted PMV could target DCs in vivo, we injected mice s.c. into the hind footpad with fluorescein-labeled PMV engrafted with ScFv, and then incubated cells isolated from the draining popliteal lymph node with a biotinylated CD11c mAb as a DC marker and streptavidin-rhodamine, after incubation with biotinylated CD11c mAb and streptavidin-rhodamine. The results show that the vast majority of these cells being CD11c+/CD14−, whereas a considerable number of fluorescein-positive cells was seen in mice injected with ScFv-engrafted PMV (~3.8%; see Fig. 2B, panels i–ii), with the vast majority of these cells being CD11c+. We also carried out analogous studies in which sections of the draining lymph node were examined by confocal scanning laser microscopy, after incubation with biotinylated CD11c mAb and streptavidin-rhodamine. The results show that compared with the control (Fig. 2C, panels i–ii), virtually all of the CD11c+ cells in the draining lymph node from mice injected with ScFv-engrafted PMV exhibit fluorescein fluorescence (Fig. 2B, panels iii–vi). The findings show that ScFv-engrafted PMV can target DCs in vivo.

Liposome-Mediated Targeting of Antigens to DCs Induces Potent Tumor-Specific Immunity Both in Vitro and in Vivo. To determine whether antigen-bearing PMV and SL targeted to DCs can be used to induce functional responses in vivo, we initially examined the ability of ScFv-engrafted PMV and SL to stimulate antigen-specific CTL responses. Recent studies have demonstrated the importance of danger/inflammatory signals during antigen exposure and DC maturation (14, 15, 36) in determining the type of immune response initiated by DCs. Thus, although the studies presented above showed that engrafted liposomes and PMV can target antigen to DCs in vitro, previous studies suggest that for this approach to induce an immune response in vivo, the codelivery of a maturation and/or danger signal to DCs also is required. To deliver both antigen and a danger signal to DCs simultaneously, we produced antigen-bearing modified PMV and SL that contained incorporated LPS, IFN-γ, or GM-CSF.

It is well known that LPS, a bacterial cell-wall component, can incorporate into lipid membranes. In preliminary studies we showed that the incorporation of LPS (1%) into SL can increase their binding to FSDC, suggesting that at least some of the liposome-associated LPS can interact with DC surface receptors, consistent with previous observations (37). In addition, evidence suggests that liposome-associated IFN-γ is biologically active and that IFN-γ can be encapsulated within liposomes and as well as can bind to the outer surface of liposomes (38, 39). Similar findings have been reported with GM-CSF (40). These attributes permit liposome-associated LPS, GM-CSF, and IFN-γ to interact with DC surface receptors and also provide the possibility that upon the binding, disruption, and/or fusion of the targeted SL or PMV, with the membrane of DCs, the SL-encapsulated agent that is released is then able to interact with receptors on DCs. Experiments also indicated that up to 1% of LPS could be included in the lipid mixture and that PMV and SL could be made to incorporate GM-CSF and IFN-γ with high efficiency, without significantly interfering with the ability of ScFv-engrafted SL to target DCs in vitro, as assessed by binding studies using flow cytometry (data not shown). Moreover, similar to what has been reported for the soluble cytokines (41), proliferation studies with FSDC showed that SL-incorporated GM-CSF induces the proliferation of FSDC in serum-free medium, whereas SL-incorporated IFN-γ inhibits proliferation in complete medium (data not shown). The results thus show that these liposome-associated cytokines are functional. Importantly, FSDC proliferation assays also could be used to monitor cytokine incorporation in the SL, which correlated well with the amounts of “free” versus SL-associated cytokine fractions as determined by SDS-PAGE analysis of fractions.

CD11c mAb N418 or the anti-DEC-205 mAb NLDC145, but not an isotype-matched control mAb, inhibited binding of the respective ScFv-engrafted SL or PMV by ~90% (see Table 2), demonstrating binding specificity for the engrafted ScFv.

Fig. 1. A, structure of the novel chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA3-DTDA). B, schematic representation of the NTA3-DTDA lipid incorporated in antigen (Ag) containing stealth liposomes (SL) composed of palmitoyl-oleoyl-phosphatidylcholine (POPC) and phosphatidyl-ethanolamine-polyethylene glycol2000 (PE-PEG2000). C, liposomes of similar composition but without PE-PEG2000 can be fused with antigen-bearing tumor cell-derived plasma membrane vesicles (PMV). In both instances, SL (B) and modified PMV (C), the lipid tracer 2-(4,4-difluoro-5-octyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-L-hexadecanoyl-phosphatidyl-choline (not shown) also can be included to facilitate tracking of either the liposomes or the modified PMV. The NTA3-DTDA permits the engraftment of histidine-tagged single chain Fv antibody fragment (ScFv) antibodies against DEC-205 and CD11c onto the liposome or modified PMV surface, and consequently, the targeting of these to surface markers such as DEC-205 and CD11c on dendritic cells.
separated by gel filtration. The results showed that typically >85% of the GM-CSF and >75% of IFN-γ became associated with the SL under the conditions used (data not shown).

To determine whether DC-targeted PMV or antigen-containing SL could generate CTL responses in vivo, we immunized C57BL/6 mice i.v. with preparations that either lacked or contained danger/maturation signals such as LPS, IFN-γ, or GM-CSF. We then isolated splenic T cells, restimulated the cells in vitro with γ-irradiated B16-OVA tumor cells, and assessed their cytolytic activity toward B16-OVA cells in a standard 51Cr release assay. The T-cell preparations used in the CTL assays against the B16-OVA were not depleted of DCs, and, hence, it would be expected that cross-presentation of B16-OVA-derived antigens can occur in vitro via these cells. Representative lytic curves are shown in Fig. 3A for animals that were immunized with various PMV preparations engrafted with the DEC-205-ScFv. The data show that little CTL activity can be detected when mice were preimmunized with PMV engrafted with the L2 peptide or with DEC-205-ScFv in the absence of a danger/maturation signal (Fig. 3A). The incorporation of either LPS or IFN-γ in the DEC-205-ScFv-engrafted PMV, however, resulted in the induction of high levels of cytolytic activity, with 50% specific lysis of target cells still occurring at a 1:1 E:T ratio (Fig. 3A). In contrast, GM-CSF was a much less effective inducer of CTL activity.

For ease of comparison, the cytolytic activity of the various PMV and SL immunization conditions are presented at the 25:1 E:T ratio in Fig. 3B. Maximum CTL activity was observed with splenic T cells from mice immunized with PMV or SL (SIINFEKL or OVA bearing) containing IFN-γ or LPS as the danger molecule. CD11c-ScFv-engrafted PMV and SL were somewhat less immunogenic, with GM-CSF being generally a less effective maturation or danger signal than IFN-γ or LPS but, nevertheless, inducing significant CTL activity when associated with PMV and OVA containing SL. Interestingly, cultures containing T cells from animals injected with ScFv-engrafted PMV or SL lacking an associated maturation or danger signal, gave near background levels of lysis (Fig. 3, A and B). In parallel experiments we tested B16, EL-4, and EL-4-OVA cells, instead of B16-OVA cells, as targets. Lytic activity against EL-4-OVA cells was higher than against B16 or EL-4 cells, but significantly lower (3–4-fold) than against B16-OVA cells (data not shown). The cytolytic response generated by ScFv-engrafted PMV and SL, therefore,
was specific for OVA and possibly antigens unique to the B16-OVA cell line.

**Liposome-Based Vaccines That Target DCs Induce Protective Immunity Against Tumors.** Mice immunized with the various B16-OVA preparations were examined for their ability to resist an i.v. challenge of B16-OVA tumor cells, with lung metastases being quantified 16 days after tumor cell injection. Compared with control mice, a much lower number of metastases was observed in mice immunized with PMV or OVA-bearing SL engrafted with ScFv and containing either LPS or IFN-γ (Fig. 4A). Fig. 4B shows representative images of the lungs from mice vaccinated with PMV containing IFN-γ and engrafted with either L2 peptide (Fig. 4B, panel i), or with DEC-205 ScFv (Fig. 4B, panel ii), from which clear differences in the appearance of the lungs can be seen. In experiments where the PMV or OVA-bearing SL were not engrafted with a ScFv and/or did not contain LPS or IFN-γ little protection to tumor cell challenge was detected. In stark contrast, SIINFEKL containing SL was unable to protect mice against tumor challenge (Fig. 4A), despite some of the vaccine constructs inducing potent CTL activity (Fig. 3). These data are consistent with the B16-OVA being resistant to clearance by CD8+ CTLs to this epitope (35).

To explore the effect of vaccination on pre-existing tumors, we injected mice with DEC-205-ScFv-engrafted PMV containing IFN-γ at day 3 and also at day 6 and day 9, after challenge with $1.5 \times 10^5$ B16-OVA tumor cells. Interestingly, vaccinated mice subsequently did not show any signs of tumor development up to 8 months after tumor challenge, whereas control animals had to be euthanized at day 22 due to an increasing tumor burden in the lungs, which contained an average of 250 ± 37 tumor foci.

The inhibition of tumor growth observed by targeting ScFv-engrafted PMV or SL containing antigen (and encapsulated with IFN-γ

Table 2  Specificity of the binding of PMV or SL, engrafted with either CD11c-ScFv or DEC-205-ScFv, to LTC-DCs

<table>
<thead>
<tr>
<th>Liposome type and specificity</th>
<th>Blocking mAb</th>
<th>Percent of control binding</th>
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<tbody>
<tr>
<td>PMV-CD1ic</td>
<td>Control</td>
<td>91</td>
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<tr>
<td>PMV-CD1ic</td>
<td>CD11c</td>
<td>10</td>
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<td>SL-CD11c</td>
<td>CD11c</td>
<td>13</td>
</tr>
<tr>
<td>SL-DEC-205</td>
<td>Control</td>
<td>94</td>
</tr>
<tr>
<td>SL-DEC-205</td>
<td>DEC-205</td>
<td>14</td>
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* PMV, plasma membrane vesicle; SL, stealth liposome; LTC, long-term culture; DC, dendritic cell; mAb, monoclonal antibody.

Fig. 3. Vaccination of mice with plasma membrane vesicles (PMV) and stealth liposome (SL) stimulates CTL activity against tumor cells. A, CTL activity of splenocytes stimulated for 4 days with γ-irradiated B16-OVA cells and derived from mice injected with PBS alone, B16-OVA PMV engrafted with L2 peptide (PMV-L2), PMV engrafted with DEC-205 single chain full-length variable antibody fragments alone (PMV-DEC-205) or in combination with lipopolysaccharide (LPS; PMV-LPS-DEC-205), IFN-γ (PMV-IFN-γ-DEC-205), or granulocyte macrophage colony-stimulating factor (GM-CSF; PMV-GM-CSF-DEC-205) B, the CTL activity of splenocytes (25:1 E:T ratio) from mice after immunization with PMV, SIINFEKL-containing SL, and OVA-containing SL, each engrafted with L2, CD11cScFv, or DEC-205-ScFv, as indicated. Results for conditions in which LPS, IFN-γ, and GM-CSF were incorporated with the engrafted PMV and SL, as indicated, also are shown.* indicate that CTL activity is significantly higher ($n = 6$, $*$, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$) than mice immunized with a corresponding antigen preparation engrafted with L2 peptide. In A and B specific lysis, at the indicated E:T ratios, was assessed in a standard ³¹Cr release assay. Results are expressed as the percentage specific lysis; bars, ±SE.
or LPS) to DCs in vivo raised the question of the mechanism by which the antitumor effect is induced. In vitro studies in which splenic DCs isolated from C57BL/6 mice were pulsed separately with different PMV and OVA-SL preparations indicated that in addition to antigen, engrafted ScFv (either CD11c or DEC-205) was essential to induce functional antigen presentation, which led to the proliferation of both CD4 and CD8 T cells, almost to the same extent (data not shown). Also, in these studies SIINFEKL-SL stimulated proliferation primarily of CD8 T cells, as expected. Because CD4 T cells have been implicated recently in the clearance of B16-OVA melanoma lung metastases through a mechanism involving the eosinophil chemokine eotaxin (35), the possibility that eotaxin also plays a role in the antitumor effects seen by targeting antigen directly to DCs in vivo was tested. For these studies eotaxin knockout mice were immunized with different vaccine preparations (either controls or PMV-DEC-205) before assessing the CTL activity of their splenic T cells toward B16-OVA cells as targets and assessing tumor growth in mice inoculated with the B16-OVA tumor. The results show that whereas the CTL activity of T cells from normal and eotaxin knockout mice are essentially identical (Fig. 5A), eotaxin knockout mice immunized with PMV containing IFN-γ and engrafted with either L2 control peptide (panel i) or DEC-205 ScFv (panel ii). This supports a role for eotaxin in inhibiting tumor growth in this system.

**DISCUSSION**

Here we report a novel strategy for DC-based tumor immunotherapy involving direct targeting of antigen to DCs in vivo. Liposomes have been hailed as having high therapeutic potential, but their use has been hampered by a lack of a simple method for attachment of targeting molecules (22, 23, 33). The novel chelator-lipid, NTA-3-DTDA (Fig. 1A), when incorporated into either SLs or into tumor cell-derived PMV (B16-OVA), enables the stable engraftment of hexa-histidine-tagged ScFv that target surface molecules on DCs (Fig. 1, B and C). The present work shows that PMV and SLs engrafted with ScFv specific for the DC markers CD11c and DEC-205 bind specifically to DCs in vitro (Table 2) and, based on flow cytometry and confocal microscopy studies, can target associated antigens directly to DCs in vivo (Fig. 2). Whereas hybrid Abs, or mAb conjugated with antigens, have been used to target DCs (9, 10), to our knowledge the present study is the first to demonstrate that DCs can be targeted using ScFv engrafted onto a membrane system.

Evidence suggests that maturation and/or “danger” signals are important in the maturation and migration of DCs after antigen exposure, and can avoid induction of tolerance to the presented antigen (14, 15, 36). Such signals are generally not required in in vitro antigen presentation assays, presumably because the DCs are “per-
and IFN-/H9253-dependent on the presence of a maturation or "danger" signal, with LPS containing IFN-/H9253-treating the greatest response (Fig. 3). Both xenogeneic tumor cells after restimulation with tumor cells in vitro elicit a strong inhibition in the growth and metastasis of the B16-OVA tumor in vivo. An additionally significant finding was the fact that, unlike control mice, which all developed severe lung metastases, mice that had been vaccinated with DEC-205-ScFv-engrafted PMV containing IFN-/H9253 after challenge with B16-OVA tumor cells subsequently did not show any signs of tumor development, indicating that the DC targeting vaccine has therapeutic activity. This therapeutic effect has been maintained for at least 8 months, and there is no evidence of tumors in the surviving mice, demonstrating a clear therapeutic potential for the use of the novel DC-targeted vaccine to elicit in vivo antitumor responses.

A particularly intriguing aspect of this study is that the apparent generation of CTL activity against the B16-OVA melanoma was not associated with tumor protection. This point is particularly evident with the SIINFEKL-SL vaccine that would be expected to generate only a CD8+ CTL response against OVA produced by the B16-OVA tumor cells. Despite the vaccine inducing a strong in vitro recall CTL response against B16-OVA tumor cells, no in vivo protection against the tumor was afforded by the immunization. One possibility is that this apparent discrepancy reflects an involvement of other tumor cell-derived antigenic peptides, in the case of the PMV vaccine, affording protection against the tumor. In contrast, the fact that the OVA-SL vaccine also induced a comparable recall CTL response to the tumor (compared with the SIINFEKL-SL preparation) implies that a CD4+ T-cell response may be mediating tumor protection (as OVA also contains CD4+ T-cell epitopes).

It is known that the B16-OVA melanoma line expresses very low levels of MHC class I and, consequently, is resistant to CTL lysis unless high avidity CTLs are used (35). The fact that T cells from mice immunized with DC targeting preparations of PMV or SL could lyse B16-OVA melanoma cells after restimulation is consistent with this interpretation, previous studies in fact indicate that CD4+ rather than CD8+ T cells are effective against B16-OVA metastases, with CD4+ T cells with a cytokine profile characteristic of T-helper 2 cells being particularly effective (35).

To explore a possible role of CD4+ T cell-mediated eosinophil recruitment in the antitumor effects induced by targeting antigen directly to DCs in this way can be effective in stimulating antitumor responses, and highlights the importance of maturation and/or "danger" signals in the induction of these responses (Fig. 3). Moreover, the finding that ScFv-engrafted SL containing SIINFEKL-6H can induce a significant cytotoxic response (Fig. 3) shows that the approach using NTA2-DTDA-containing SLs may be an effective strategy for targeting any histidine-tagged peptide antigen to DCs in vivo.

A finding of paramount importance in this work was our observation that syngeneic animals immunized with CD11c-ScFv- and DEC-205-ScFv-engrafted PMV or DEC-205-ScFv-engrafted PMV had much lower numbers of tumor metastases in their lungs compared with controls, after challenge with the B16-OVA melanoma. Similarly, syngeneic animals immunized with ScFv-engrafted SL containing OVA and either LPS or IFN-/H9253 had substantially lower numbers of metastases (Fig. 4). The results additionally show that tumor immunity was completely dependent on the presence of the maturation or "danger" signals, LPS and IFN-/H9253(Figs. 3 and 4). The immunization of mice with CD11c-ScFv- and DEC-205-ScFv-engrafted PMV and antigen-bearing SL, therefore, target the associated antigen(s) to DCs, which then process and present the antigens to T cells inducing antigen-specific T-cell activation and elicit a strong inhibition in the growth and metastasis of the B16-OVA tumor in vivo. An additionally significant finding was the fact that, unlike control mice, which all developed severe lung metastases, mice that had been vaccinated with DEC-205-ScFv-engrafted PMV containing IFN-/H9253 after challenge with B16-OVA tumor cells subsequently did not show any signs of tumor development, indicating that the DC targeting vaccine has therapeutic activity. This therapeutic effect has been maintained for at least 8 months, and there is no evidence of tumors in the surviving mice, demonstrating a clear therapeutic potential for the use of the novel DC-targeted vaccine to elicit in vivo antitumor responses.

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To explore a possible role of CD4+ T cell-mediated eosinophil recruitment in the antitumor effects induced by targeting antigen directly to
DC in vivo, ectopic knockout mice were immunized with ScFv-engraved PMV before inoculating them with the B16-OVA tumor. The results show that compared with controls, ectopic knockout mice exhibit a markedly reduced ability to inhibit the growth and metastasis of the B16-OVA tumor (Fig. 5). Ectopic is a potent eosinophil chemokine and, therefore, the findings are consistent with the recruitment of eosinophils into the tumor constituting an important component of the antitumor response. Whereas previous studies showed that ectopic plays a role in the elimination of B16-OVA lung metastases after the adoptive transfer of CD4+ T cells (35), the present study shows clearly that the antitumor effect elicited by vaccination with the novel DC-targeted vaccine is at least partially dependent on ectopic, thereby providing some insight into the mechanism by which the DC-targeted vaccine elicits its antitumor effects. The fact that vaccination offers partial protection in ectopic knockout mice (Fig. 5) suggests that other mechanisms, presumably dependent on CTLs, are also involved.

In summary, the modified PMV and SL system described herein offers a number of advantages over current strategies using DCs for tumor immunotherapy. Firstly, the system can deliver antigens directly to DCs in vivo, thus eliminating the need to isolate DCs from patients and to manipulate the cells ex vivo for use in immunotherapies. Secondly, a targeted or active liposome-mediated delivery of antigen to DCs has the potential to deliver more antigen and/or several different antigens, simultaneously, potentially stimulating a more effective immune response. The same approach could potentially deliver to DCs any antigen or immunostimulatory agent, such as “danger” signals, RNA, DNA, and cytokines, or combinations thereof, which cannot be easily achieved using antigens fused to DC targeting proteins (14, 15). Thirdly, the approach is versatile and would be convenient to use clinically, because potentially any DC targeting protein(s) possessing a histidine tag can be conjugated onto the modified PMV or SL to deliver specific tumor antigens or other agents to enhance tumor immunity in patients.

REFERENCES

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Targeting Dendritic Cells with Antigen-Containing Liposomes: A Highly Effective Procedure for Induction of Antitumor Immunity and for Tumor Immunotherapy

Christina L. van Broekhoven, Christopher R. Parish, Caroline Demangel, et al.


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