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Intratumoral Delivery of Antigen with Complement C3-bound Liposomes Reduces Tumor Growth in Mice

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Abstract

Antigen presenting cells (APCs) initiate the immune response against cancer by engulfing and presenting tumor antigen to T cells. Our lab has recently developed a liposomal nanoparticle that binds complement C3 proteins, allowing it to bind to the complement C3 receptors of APCs and directly deliver antigenic peptides. APCs were shown to internalize and process complement C3-bound liposomes containing ovalbumin (OVA), resulting in a significant increase in activated T cells that recognize OVA. Mice bearing A20-OVA lymphoma tumors were treated with OVA-loaded C3-liposomes, which led to reduced tumor growth in both treated and distal tumors in all mice. Peripheral blood from treated mice had a lower percentage of immunosuppressive myeloid derived suppressor cells (MDSCs), a higher percentage of B cells, and increased anti-OVA IgG₁ levels compared to control mice. These results indicate that C3-liposome delivery of tumor antigen to APCs initiates a potent and systemic antitumor immune response.

Graphical abstract summary

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OPSS (orthopyridyl disulfide) groups within liposome membranes bind activated complement C3 serum proteins, forming C3-bound liposomes. C3-liposomes are specifically targeted to immune cells that carry the receptor for C3 proteins, such as antigen presenting cells (APCs). APCs internalize C3-liposomes through C3 receptors, which are then transported to endosomes for processing. Encapsulated antigens (ovalbumin) are cleaved into peptide fragments and loaded onto major histocompatibility complexes (MHC) for presentation on the external cell surface.

Keywords

antigen-presenting cells; cancer immunotherapy; complement C3; nanoparticle; targeted delivery; tumor antigen

BACKGROUND

Tumor antigens are proteins that provide specific targets for CD8⁺ T cells (cytotoxic T lymphocytes: CTLs), allowing the immune system to distinguish cancer cells from noncancerous cells.^{1–3} Tumor antigens can be mutated peptides, proteins expressed by genes which are normally silent, cancer-germline antigens, which are only present on tumor cells, or viral epitopes, present on virus-associated tumors.^{1, 4} Alternatively, they can be normal proteins expressed at a higher degree on tumor cells, but still present in normal tissue (overexpressed or differentiation antigens).^{1, 4} Regardless of the type of antigen, antigenic activation is essential for the success of cancer immunotherapies.

The goal of a tumor vaccine is to improve T cell recognition of tumor antigens. Tumor vaccines can be derived from a single tumor antigen, an antigenic epitope, or multiple antigens for a given tumor.^{1, 3-6} Using multiple antigenic epitopes is advantageous due to the occurrence of immunoediting, whereby cancer cells limit the expression of certain antigens in order to hinder immune surveillance and allow for immune escape.^{7, 8} The presence of multiple lineages of CTLs with receptors specific for different antigens creates a persistent attack on tumor cells, even in the presence of tumor-mediated antigen downregulation.

T cells must encounter a certain threshold of antigen presentation to overcome the natural tolerance mechanisms in place to prevent autoimmunity and acute inflammation.⁹⁻¹¹ This is especially relevant when working with tumor antigens derived from over-expressed or differentiated antigen variants, due to their expression in normal tissue.^{4, 5} Targeted liposome nanoparticles are an effective means of increasing the amount of antigen delivered as well as increasing the specificity of delivery to APCs.^{12, 13} In addition, liposomes can encapsulate multiple antigens simultaneously to strengthen the immune response against a tumor.^{1, 4, 14} Other strategies for effective tumor vaccines often involve *ex vivo* proliferation and treatment of autologous dendritic cells (DCs), followed by re-infusion into the patient, akin to adoptive T cell transfers. In contrast, targeted liposomes would allow for *in vivo* delivery of antigenic peptides to APCs without the need for costly *ex vivo* culturing and re-infusion into the patient.¹⁵⁻¹⁷

Many liposome systems have been developed to target antigen presenting cells, such as cationic, mannose, Fc-targeted, CD11c-targeted, and DC-SIGN-targeted liposomes.^{13, 18} Many of these systems require complex targeting molecules, antibodies or cationic lipids which can be associated with high levels of toxicity.^{12, 13} We have developed a liposome nanoparticle (C3-liposomes) that utilizes neutral lipids and endogenous serum proteins, thereby reducing both toxicity from cationic lipids and immunogenicity of foreign proteins while decreasing expense associated with targeting antibodies and ligands.^{19, 20} Most importantly, C3-liposomes contain a pegylated lipid with an exposed orthopyridyl disulfide (OPSS) group that can disulfide bond with the unique sulfhydryl group on complement C3b, which is exposed when complement C3 protein is activated to C3b.²¹ By virtue of covalently-bound complement proteins, C3-liposomes can specifically target a range of immune cells that carry the receptors for activated complement C3 derivatives. These receptors are expressed primarily by myeloid cells, including macrophages and dendritic cells, as well as by B cells.²²⁻²⁴ We previously showed that C3-liposomes are internalized by all myeloid cell types, providing a unique delivery device to APCs.^{19, 20}

To assess the potential of using C3-liposomes with encapsulated antigen as a tumor vaccine, we tested the ability of C3-liposomes to deliver the mock tumor antigen ovalbumin (OVA) to APCs and activate DO11.10 T cells *in vitro*. OVA, a protein in egg whites, is commonly used as a mock antigen to study antigen-specific immune responses in mouse models. Reporter DO11.10 T cells express GFP and fluoresce green upon binding OVA peptide-MHCII complexes presented by APCs²⁵. These studies were followed by *in vivo* experiments in mice with A20-OVA tumors, whereby C3-liposomes with encapsulated OVA

were shown to deliver tumor antigen, activate an antigen-specific immune response, and reduce growth of established tumors.

METHODS

Reagents

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000] (DSPE-PEG(2000)), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[PDP-poly(ethylene glycol)-2000] (DSPE-PEG(2000)-PDP) for liposome preparation were purchased from Avanti Polar Lipids (Alabaster, AL). Consistent with our previous publications, we use the term OPSS to refer to the PDP group. Fluorescently tagged lipid, Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (RhodaminePE), was purchased from Life Technologies (Grand Island, NY, USA). Size exclusion chromatography used CL-4B Sepharose gel, purchased from Sigma-Aldrich (St. Louis, MO, USA). Human serum with complement C3, and serum depleted of complement C3 were obtained from Quidel Corporation (Athens, OH, USA). Anti-human antibodies, PE/Dazzle 594 CD3, PerCP/Cy5.5 HLA-DR, APC CD14, Alexa Fluor 700 CD11c, APC/Cy7 CD11b, Pacific Blue CD15, Brilliant Violet 650 CD20, Brilliant Violet 605 CD33, Brilliant Violet 785 CD56, and anti-mouse antibodies, FITC CD45, PE CD25, PE/Dazzle 594 CD19, PerCP Ly-6G, PE/Cy7 CD11c, APC CD3, Alexa Fluor 700 CD11b, APC/Cy7 CD8b, Brilliant Violet 421 FOXP3, Brilliant Violet 510 Ly-6C, Brilliant Violet 605 IA/IE, Brilliant Violet 650 F4/80, Brilliant Violet 785 CD4, were purchased from BioLegend (San Diego, CA, USA). Anti-human antibody, PC7 CD45, was purchased from Beckman Coulter (Brea, CA, USA). All other chemicals, reagents, and kits were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).

Cell lines

The A20-OVA cell line was kindly provided by Dr. Gang Zhou (Augusta University, Atlanta, Georgia). This lymphoma tumor cell line was stably transfected with a construct coding for membrane-bound ovalbumin as a mock tumor antigen²⁶. The reporter T cell line, I-A^d-restricted OVA-specific murine T cell hybridoma DO11.10, was a generous gift from Dr. David Underhill (UCLA, Los Angeles, CA). Tumor cells and T cells were cultured in culture medium (RPMI, 10% heat inactivated FBS, 1% penicillin/streptomycin) supplemented with 0.05 mM 2-mercaptoethanol and incubated at 37°C in 5% CO₂.

Liposome preparation

Liposomes were prepared using a previously described film hydration method.^{20, 27} OPSS liposomes were made by mixing DPPC/DSPC/DSPE-PEG(2000)-PDP/DSPE-PEG(2000)/RhodaminePE in chloroform at a molecular ratio of 83:12:1:3:1. Control-liposomes were made following the same procedure, substituting DSPE-PEG(2000)-PDP with DSPE-PEG(2000). Lipid mixtures were dried under nitrogen stream for 1 hour, followed by rehydration of the film with 0.7 mL of filtered water for non-protein encapsulated liposomes. Liposomes containing ovalbumin (OVA) were rehydrated with 0.7 mL 80 mg/mL ovalbumin solution and liposomes containing fluorescent DQ-OVA were rehydrated in 0.7 mL of 1

mg/mL DQ-OVA solution. Liposomes were extruded 9 times through a 400 nm polycarbonate membrane filter at 47°C. Extruded liposomes were column purified using a CL-4B sepharose column hydrated in 1× PBS, pH 7.4. The concentrations of control- and OPSS-liposome samples were normalized using a NanoDrop 2000 UV-Vis spectrophotometer, observing the rhodamine peak and diluting to a lipid concentration of 0.875 mg lipid/mL. Liposome size was determined using a Malvern Zetasizer Nano-S (Malvern Instruments, Malvern, UK); (control-liposomes: 262.1 ± 65.74 nm, OPSS-liposomes: 265.4 ± 101.6 nm). Encapsulation efficiency was determined by encapsulation of Alexa Fluor 488-OVA (1 mg/ml) and OVA (79 mg/ml) for a final concentration of 80 mg/ml. After column purification, rhodamine fluorescence was used to determine liposomal concentration in the peak collected fraction and Alexa Fluor 488 fluorescence intensity was used to determine the level of OVA encapsulation. Encapsulation efficiency was estimated at 5.5%. The peak fraction of collected liposomes had a 1:240 dilution of OVA compared to the rehydration solution, and this dilution was used to match control levels of non-encapsulated OVA in experimentation.

***In vitro* analysis of antigen processing and presentation**

Human whole blood, obtained from healthy volunteers, was collected in heparinized tubes. The blood draw protocol was approved by the UAA Institutional Review Board, in accordance with the U.S. Department of Health and Human Services requirements for the protection of human research subjects (45 CFR 46 as amended/revised), and all donors provided written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-paque gradient separation. Isolated PBMCs were re-suspended in serum-free RPMI and plated at 1.6×10^5 cells per well in a 96-well V-bottom plate.

For fluorescence microscopy, monocytes were isolated from PBMCs to enrich for antigen presenting cells. Monocyte isolation was performed by negative selection using a monocyte enrichment kit (Becton Dickinson, San Jose, CA, USA).

Antigen processing by cells was analyzed using DQ-OVA (Molecular Probes), which fluoresces green after proteolytic degradation. 10 µL of rhodamine labeled OPSS- and control-liposomes, containing DQ-OVA, were incubated in 10 µL of C3-positive and -negative serum for 1 hour prior to addition to either PBMCs or enriched monocytes. Liposomes and serum were added to cells (final serum concentration of 10%) and incubated for 3 hours at 37°C, 5% CO₂. Cells were centrifuged at 500×g for 5 minutes and rinsed twice in 1× PBS. Cells were analyzed by fluorescence microscopy and flow cytometry for liposome internalization (rhodamine) and antigen processing and presentation (DQ-OVA).

Fluorescence microscopy

Cells were transferred to a V-bottom plate, centrifuged 500×g 5 minutes, and rinsed twice with 1× PBS before transfer to a Falcon flat-bottom 96-well plate, black/clear bottom (Becton Dickinson Labware, Franklin Lakes, NJ, USA) for imaging. Photos were taken using a Leica DMI6000B inverted fluorescence microscope (Leica Microsystems, Buffalo

Grove, IL, USA) and a 10× objective utilizing Leica Application Suite, version 3.7.0 software (Leica Microsystems Inc., Wetzlar, Germany).

Flow cytometry analysis

Cellular internalization of rhodamine labeled liposomes was determined by mean fluorescence intensity (MFI) of rhodamine, detected on the PE channel. Antigen processing and presentation of DQ-OVA were determined by MFI, detected on the FITC channel. Cell types were determined by fluorescence of specific cell marker antibodies, and staining protocol and cell type selection method was followed as previously described.¹⁹

Generation of bone marrow derived dendritic cells

Bone marrow was extracted from adult BALB/c mice and plated at a density of 2×10^6 cells in culture medium. Granulocyte-macrophage colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4) were added at 40 ng/mL and 20 ng/mL, respectively. Cells were incubated at 37°C and medium (including cytokines) was replenished after 3 days. On day 6, non-adherent and loosely-adherent cells were harvested by pipetting and re-suspended in culture medium.

T cell activation

10 μ L of OPSS- and control-liposomes containing OVA, and free OVA matching the amount encapsulated in OPSS-liposomes, were incubated in 10 μ L C3-positive and -negative serum for 1 hour at room temperature prior to addition to 1.6×10^5 bone marrow-derived dendritic cells in RPMI (1% penicillin/streptomycin) in a U-bottom plate (final serum concentration was 10%). Cells were incubated with liposomes or controls for 24 hours at 37°C, 5% CO₂. Cells were rinsed twice in RPMI to remove any non-internalized liposomes and re-suspended in culture medium. A reporter T cell line, I-A^d-restricted OVA-specific T cell hybridoma DO11.10, was added to the dendritic cells at a 1:1 ratio and incubated for 24 hours. These reporter T cells are activated only by APCs presenting OVA peptides and express GFP when activated. The co-cultures were then analyzed for T cell activation (GFP fluorescence) by fluorescence microscopy and flow cytometry.

A20-OVA tumor inoculation and mouse model

Female and male 6–10 week old BALB/c mice were obtained from The Jackson Laboratory (Sacramento, CA, USA) and housed in the University of Alaska Anchorage (UAA) vivarium. All experiments were approved by the UAA Institutional Animal Care and Use Committee. A20-OVA cells were rinsed twice in 1× PBS. Mice were shaved and received subcutaneous injections in their left and right flanks, 1.5×10^6 A20-OVA cells in 25 μ L of PBS per injection; injections were given under anesthesia using isoflurane. Treatments were started when tumors became palpable (approximately 10–14 days). Mice were separated into groups of 4–5 and received local subcutaneous injections of 100 μ L of either 1× PBS, non-encapsulated OVA equal to the encapsulated amount, control-liposomes containing OVA, OPSS-liposomes containing OVA, or OPSS-liposomes without OVA. Mice received treatment on one side only. The tumor on the opposite side was measured to document systemic response to treatment. Injections were given on days 1, 2, 4, 6, 8, 10, and 12 (day 1

being the first injection). Tumor measurements were made before all injections and for 4 days after the final injection, using a digital caliper. Volumes were reported as mm³: $[(4/3)\pi(\text{length} \times \text{width} \times \text{minimum})/8]$. Mice were monitored daily for signs of discomfort and distress.

Analysis of mouse blood

Mice were euthanized following therapy, 4 days after the last injection, and blood was collected in heparinized tubes via cardiac puncture. Plasma was separated from blood via centrifugation at 500×g for 15 minutes and frozen at –80°C for subsequent analysis. Red blood cell lysis buffer (eBioscience) was added to the blood for 5–10 minutes at room temperature. Samples were then prepared for flow cytometry analysis or frozen in culture medium supplemented with 10% dimethyl sulfoxide for later use.

Liver toxicity assays

Mouse plasma levels of aspartate transaminase (AST) and alanine transaminase (ALT) were determined using kits purchased from BioAssay Systems (Hayward, CA, USA). Plasma samples were diluted 1:1 in assay buffer prior to addition to a Falcon flat-bottom 96-well plate, black/clear bottom (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Absorbance was measured at 340 nm at 5 and 10 minutes using a BioTek Synergy HT plate reader utilizing Gen5 software, version 2.01., and enzyme activity was calculated according to the protocol.

ELISA

Mouse plasma levels of anti-OVA IgG₁ were determined by ELISA using a kit purchased from Cayman Chemicals (Ann Arbor, MI, USA). Plasma samples were diluted 1:2000 in assay buffer prior to assay, and the provided kit procedure was followed. Absorbance was measured at 450 nm using a BioTek Synergy HT plate reader.

Statistical analysis

Data is presented as mean ± standard error (n=4–5). Differences among groups were determined using an unpaired two-tailed Student's *t* test for tumor volume measurements, and the Mann-Whitney-U test for samples with a non-normal distribution. P values of less than 0.05 were considered significant and are indicated by an asterisk over the data.

RESULTS

APCs internalize C3-liposomes and process antigen

Liposomes that contain an OPSS group have the ability to form a disulfide bond with activated complement C3 proteins, leading to uptake by antigen presenting cells (APCs) through their complement receptors.^{19, 20, 28, 29} To determine if such liposomes could deliver antigen and enhance antigen presentation compared to control liposomes without OPSS, both sets were formulated to contain an encapsulated antigen, DQ-OVA. APC uptake of liposomes was determined via a fluorescent rhodamine lipid incorporated into the membranes of both OPSS- and control-liposomes. Antigen processing of DQ-OVA was

observed through production of FITC fluorescence that occurs as the self-quenched DQ-OVA undergoes proteolytic degradation to peptides in the endosome for antigen presentation.

OPSS liposomes were incubated in human serum, containing complement C3 proteins, to produce targeted C3-bound liposomes (C3-liposomes). Liposomes lacking the OPSS group (control-liposomes) were incubated in human serum simultaneously; these liposomes do not form bonds with complement C3, creating a control, non-targeted liposome¹⁹. Additional controls included both OPSS- and control-liposomes incubated in human serum depleted of complement C3 protein, and non-encapsulated free DQ-OVA administered at the same concentration as that encapsulated in liposomes. Each treatment type was administered to Ficoll-isolated white blood cells from whole human blood.

Flow cytometry analysis of rhodamine fluorescence revealed extensive uptake of C3-liposomes by the three APC types, macrophages (CD11b⁺CD14⁺), dendritic cells (CD11c⁺) and B cells (CD19⁺) (Figure 1), confirming our previously reported results.¹⁹ In addition, DCs and macrophages showed intense FITC fluorescence, indicating proteolysis of delivered DQ-OVA (Figure 1A and 1B). Interestingly, B cells bound high levels of C3-liposomes but did not show evidence of antigen processing (Figure 1C). The high level of rhodamine and FITC in APCs was only observed when DQ-OVA antigen was delivered by C3-liposomes incubated in complete human serum. Control-liposomes without the OPSS-group and liposomes incubated in C3-depleted serum exhibited relatively low uptake by APCs. Based on these results showing the necessity of both complement C3 in serum and the OPSS group on liposomes, uptake of liposomes is attributed to complement C3 proteins bound to the liposomal OPSS-groups, which target complement receptors on the plasma membranes of APCs.

Importantly, based on FITC intensity, uptake of DQ-OVA C3-liposomes resulted in a 91-fold increase in processed DQ-OVA in macrophages and a 54-fold increase in dendritic cells, when compared to non-targeted free DQ-OVA at the same concentration (Figure 1). This difference in antigen delivery is confirmed by fluorescent microscopy, which shows isolated human monocytes exposed to rhodamine labeled C3-liposomes or control-liposomes that contain DQ-OVA, or to free DQ-OVA at the same concentration (Figure 2). The intense red rhodamine fluorescence shows the high uptake of C3-liposomes by monocytes, and the DQ-OVA antigen processing shown in green is remarkably higher when targeted within C3-liposomes.

Delivery of antigen to APCs with C3-liposomes leads to T cell activation

To evaluate the ability of C3-liposomes to deliver OVA antigen to APCs and activate an antigen-specific T cell response, OVA was delivered to bone marrow-derived dendritic cells (BMDCs), and then co-incubated with the OVA-specific reporter T cell line DO11.10. The T cell receptor on DO11.10 T cells recognizes antigenic epitopes of OVA peptides when presented by APCs. In response to APC presentation of OVA epitopes and stimulation of the T cells, GFP is expressed at a high level within the T cells, allowing for a direct measurement of OVA-specific T cell activation. GFP fluorescence was observed by means of both fluorescence microscopy and flow cytometry (Figure 3).

Co-cultures after treatment with OVA-encapsulated C3-liposomes (OVA C3-liposomes) resulted in the highest percentage of T cell activation ($68.7 \pm 2.7\%$) (Figure 3). Controls, including cultures treated with PBS, OVA-encapsulated control-liposomes (OVA control-liposomes) and the equivalent non-encapsulated free OVA displayed significantly lower levels of T cell activation, (OVA control-liposomes: $18.0 \pm 1.3\%$; free OVA: $14.4 \pm 0.8\%$; PBS: $11.3 \pm 0.1\%$). These results confirm that C3-bound liposomes improve antigen (OVA) presentation by APCs and that OVA epitopes are specifically recognized by OVA-specific T cells which are thereby activated.

Treatment with OVA C3-liposomes leads to reduced tumor growth in mice

To evaluate if C3-liposomes could deliver tumor antigen (OVA) and activate an antigen-specific immune response *in vivo*, OVA C3-liposomes were used to treat established A20-OVA lymphoma tumors in male and female BALB/c mice. A20-OVA cells express OVA as a mock tumor antigen and can be used to determine if OVA vaccination leads to reduction in tumor growth. Each mouse received a local subcutaneous injection of a specific treatment at only one tumor site while the other tumor was left untreated to gauge the systemic response to therapy. Treatment groups included PBS, OVA C3-liposomes, OVA control-liposomes, non-encapsulated free OVA, or C3-liposomes without OVA.

Mice receiving OVA C3-liposome treatments had reduced tumor growth in both the injected and distal tumors and complete rejection of the injected tumors in three out of five mice. In addition, two of these three mice eliminated both the injected and distal tumors and were cancer free at the end of the experiment (Figure 4). The reduction in tumor growth in both the injected and distal tumors indicates an effective systemic anti-tumor immunity in response to antigen delivery with C3-liposomes. All other treatment groups exhibited continuous tumor growth, with no significant difference from PBS treated mice in either the injected or the distal tumor (Figure 4). The significant reduction in tumor growth in mice treated with C3-liposomes containing OVA indicates that both the OPSS group and the tumor-specific antigen are needed to elicit an effective anti-tumor immune response.

To assess treatment toxicity, liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT) were measured in mouse plasma, since elevated levels of AST and/or ALT in blood are indicators of liver damage. Results from treatment groups are within normal ranges, with no significant differences between treatment groups (Table 1).

OVA C3-liposomes decrease MDSC percentages and increase circulating B cell percentages

Two weeks following initial treatment, mice were euthanized and blood was collected for analysis of circulating immune cells. OVA C3-liposome treated mice had significantly lower levels of systemic $CD11b^+Ly6c^{hi}$ myeloid-derived suppressor cells (MDSCs), compared to mice treated with OVA control-liposomes, C3-liposomes without OVA, free OVA, or PBS (Figure 5A). Furthermore, mice that eliminated at least one tumor in response to treatment had the lowest level of MDSCs. OVA C3-liposome treated mice that had eliminated a tumor had elevated percentages of $CD19^+$ B cells, compared to all other treatment groups (Figure 5B). No significant differences in blood T cell numbers were found between treatment

groups (Figure 5C), but this may be due to the length of time between tumor reduction and analysis of white blood cell numbers.

Treatments increase anti-OVA IgG₁

Plasma was collected from mouse blood samples to determine the level of circulating anti-OVA IgG₁ between treatment groups. ELISA analysis of plasma samples revealed significant increases in anti-OVA IgG₁ in all treatment groups compared to the PBS-treated mice (Figure 6). These results indicate that mice exposed to OVA antigen produced a humoral immune response to OVA within 14 days following the first injection. Of note, was that empty C3-liposomes also provoked a low-level antibody based immune response to OVA, indicating possible adjuvant activity of C3-liposomes.

DISCUSSION

Antigen presenting cells (APCs) initiate an immune response by processing antigens and presenting antigenic epitopes complexed with MHC class II molecules to T cells. Tumor vaccines aim to deliver tumor antigens to APCs to bolster antigen presentation and thereby enhance the immune response against cancer.^{1-3, 5, 12} As a result of patient tumor sequencing, there is a growing library of identified tumor antigens and an increasing need for technologies that can deliver tumor antigens directly to APCs.^{12, 30} For this purpose, we have designed liposomes with lipid-attached OPSS groups which form covalent bonds with activated complement C3 proteins and, as a result, are phagocytosed by APCs with receptors for C3 fragments such as iC3b, C3d, and C3dg.²⁷⁻²⁹

C3-liposomes with encapsulated tumor antigen resemble complement-coated pathogens and are taken up by all three types of APCs, resulting in efficient antigen delivery and processing in macrophages and dendritic cells.¹⁹ Compared to non-targeted antigen, C3-liposomes greatly improve uptake and proteolytic cleavage of the model antigen DQ-OVA, the first steps in initiating an adaptive immune response. Complement receptors are not generally associated with clathrin-coated pits and have been shown to internalize through a macropinocytotic pathway that depends on both receptor cross-linking and microfilaments.³¹ Upon internalization, the vesicles are routed for antigen processing and result in presentation of phagocytosed proteins by MHCII complexes. Interestingly, B cells take up high levels of C3-liposomes with DQ-OVA but do not process the encapsulated antigen. Further experimentation will be needed to determine why antigen is not processed in B cell endosomes. BMDCs targeted by C3-liposomes loaded with antigenic OVA activate T cells that display the receptor for OVA. This activation is dependent on liposome encapsulation of OVA and delivery mediated by C3 targeting. Taken together, these *in vitro* results show the ability of C3-liposomes to enhance antigen delivery to APCs, with subsequent T cell activation.

Intratumoral treatment with OVA C3-liposomes leads to tumor growth reduction that is greater than other treatments, resulting in elimination of three out of five injected tumors. To evaluate if there was a systemic immune response, tumor growth was measured at the distal site that did not receive injections. C3-liposome delivery of antigen is the only treatment that results in a systemic response, leading to reduced tumor growth and complete elimination of

distal tumors in two out of five mice. Surgical subcutaneous analysis of rejected tumor sties revealed no evidence of tumor lesions or angiogenic vessels, and skin appeared healthy in all regards.

If APCs are not properly activated to provide positive co-stimulation to T cells, they can trigger an upregulation of T regulatory cells, immune evasion, and tolerance to tumor antigens.^{32, 33} In our study, the observed reduction in tumor growth in response to OVA C3-liposomes implies that the C3-liposome delivery system is activating APCs. Additionally, mice treated with empty C3-liposomes have a low-level antibody response against OVA, albeit about 10-fold lower than mice treated with OVA C3-liposomes, indicating an antigen-specific immune response. Previous work by others has shown that complement C3 receptor stimulation leads to increased activation in macrophages and a 1000-fold reduced threshold for activation in B cells.^{34–36} It is possible that when C3-liposomes deliver OVA, they are also activating APCs through a complement-dependent mechanism.

Mice treated with OVA containing C3-liposomes have decreased levels of MDSCs, immature cells that expand in number in response to signals and cytokines released from the tumor.^{37, 38} Our previous work showed the ability of C3-liposomes to target MDSC, and it could be that delivery of antigen with C3-liposomes results in decreased MDSC¹⁹. Alternatively, the decrease in MDSCs could be due to a reduction in overall tumor burden in mice treated with OVA C3-liposomes. Another possibility, is that mice with inherently low levels of MDSCs are more responsive to treatment, given that the mice observed with complete tumor rejection had the lowest level of MDSCs. Further experimentation will be needed to fully elucidate the relationship between C3-liposome treatment and MDSC levels. In cancer patients, MDSCs are a key cell type responsible for promoting immunosuppression, such that elevated systemic levels correlate with cancer progression and poor prognosis.^{39, 40} If C3-liposomes can impact MDSC immunosuppression, they could provide an important mechanism for improving immunotherapy.

Even with successful antigen delivery to APCs and subsequent T cell activation by C3-liposomes, the effectiveness of an immune response could be limited by T regulatory cells, MDSCs and tumor cell expression of PD-L1. Therefore, treatment with C3-liposomes may be most effective if used in combination with existing immunotherapies. Two antibody-based cancer immunotherapies, anti-PD-1 and anti-CTLA-4, have had success in treating melanoma, among other cancers.^{41, 42} CTLA-4 is a receptor located on T regulatory cells and is responsible for blocking the interaction between APCs and T cells.⁴³ PD-1 is a receptor located on T cells that results in T cell anergy or apoptosis when bound by its ligand (PD-L1), which is commonly upregulated in tumor cells.^{42, 44} The combination of increased antigen presentation and reduced number of MDSCs resulting from C3-liposome treatment, along with reduction of T regulatory cells by anti-CTLA-4 and less T cell anergy due to anti-PD-1 treatment, could result in a powerful anti-tumor immune response.

Immunotherapies are often limited by autoimmunity and other toxicities associated with the treatment. C3-liposomes are composed of neutral lipids and have a polyethylene glycol layer that reduces aggregation and results in minimal toxicity, as revealed by normal AST and ALT liver enzymatic levels and by no evidence of pulmonary distress after treatment. C3-

liposomes bind to endogenous complement C3 in the blood, thereby negating unwanted immunogenicity due to foreign targeting ligands. In addition, C3-liposomes could provide a cost-efficient means of treatment, without the need for labor intensive *ex vivo* cultures, expensive patient-specific reagents, or immunoglobulin-based targeting.

The results described here demonstrate the potential of C3-liposomes for improving antigen delivery and T cell activation. Further *in vivo* treatment of tumors with C3-liposomes will focus on delivering tumor antigens derived from spontaneous mutations in mouse tumor cell lines and on testing C3-liposome treatment in combination with anti-CTLA-4 and anti-PD-1 immunotherapies. With a growing library of known tumor antigens, C3-liposomes may provide an important technology for enhancing cancer immunotherapy.

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Figure 1. C3-liposomes improve antigen delivery to monocytic APCs.

Rhodamine-labeled OPSS-liposomes containing DQ-OVA incubated in human serum containing complement C3 (C3-liposomes) are taken up by (A) macrophages, (B) dendritic cells, and (C) B cells. Rhodamine-labeled control-liposomes (liposomes that contain DQ-OVA but without OPSS) and OPSS-liposomes lacking complement C3 are not taken up by cells. When compared with an equal amount of non-encapsulated DQ-OVA (Free DQ-OVA), C3-liposomes improve uptake and processing of DQ-OVA in macrophages and dendritic

cells. B cells internalize C3-liposomes, but do not process DQ-OVA. Data are expressed as mean $\pm$ standard error (n=3). *p-value < 0.05, compared to Free DQ-OVA.

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Figure 2. C3-liposomes increase delivery and processing of DQ-OVA.

Human monocytes were incubated with rhodamine-labeled C3-liposomes, control-liposomes containing DQ-OVA, or free DQ-OVA for 3 hours. Monocytes were rinsed and imaged for rhodamine-labeled liposomes and for DQ-OVA, which fluoresces as FITC when processed for presentation. DQ-OVA C3-liposomes show high uptake into monocytes (rhodamine) and improved delivery of DQ-OVA compared to DQ-OVA control-liposomes (without OPSS) and non-encapsulated DQ-OVA at the same concentration (DQ-OVA Free).

Figure 3. C3-liposomes targeting BMDCs increase T cell activation.

Bone marrow-derived dendritic cells were incubated with rhodamine-labeled C3-liposomes containing OVA for 24 hours (OVA C3-liposomes). Dendritic cells were rinsed, and T cells engineered to express GFP when presented with OVA epitopes were co-cultured with the dendritic cells for 24 hours. C3-liposome treatment resulted in increased T cell activation, compared to control-liposomes containing OVA (OVA control-liposomes) or an equivalent amount of non-encapsulated OVA (free OVA). Data are expressed as mean $\pm$ standard error (n=3). *p-value < 0.05, compared to PBS.

Figure 4. OVA C3-liposomes result in reduced tumor volume of both treated and distal established tumors.

Established A20-OVA tumors were treated by intra-tumor injections on days 1 & 2, and then every other day for a total of 7 injections. Tumor measurements were made before all injections, showing (A) Injected tumor volume, and (B) Non-injected distal tumor volume (mm³). By day 8, and through the end of the study, only OVA C3-liposomes significantly reduced injected and distal tumor volumes (*p-value < 0.05, compared to PBS). No other treatment groups resulted in significant reduction of tumor volume compared to PBS. Injected and distal tumors treated with OVA C3-liposomes are significantly smaller (p-value

< 0.05) than those treated with free OVA and OVA control-liposomes (w/o OPSS) from day 8 onward, but for the sake of clarity this is not shown in the figure. Data are expressed as mean $\pm$ standard error (n=4–5).

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Figure 5. OVA C3-liposome treatments result in a lower percentage of MDSCs and a higher percentage of B cells.

Mice from the tumor treatment groups were euthanized and peripheral blood collected. Percentages of MDSCs (CD11b⁺, Ly6C^{high}), B cells (CD19⁺), and T cells (CD3⁺) were analyzed by flow cytometry. Cell populations are displayed as percentages of the total number of white blood cells. Data are expressed as mean $\pm$ standard error (n=4–5). *p-value < 0.05, compared to PBS.

Figure 6. Treatments result in higher circulating levels of anti-OVA IgG₁.

Mice from the tumor treatment groups were euthanized and plasma collected. Anti-OVA IgG₁ levels were analyzed via ELISA. Data are expressed as mean $\pm$ standard error (n=4–5).

*p-value < 0.05, compared to PBS.

Table 1.

Liver enzyme activity in mouse plasma samples

Treatment Group	ALT* (U/L)	AST [†] (U/L)
OVA Control-liposomes	71.7 ± 23.8	88.4 ± 40.9
OVA C3-liposomes	60.3 ± 19.5	171.0 ± 42.6
Free OVA	51.2 ± 34.7	110.4 ± 25.6
PBS	40.6 ± 26.4	82.6 ± 18.5
Normal range [‡]	15–84	54–298

Data are presented as mean ± SD (n=3)

* alanine transaminase

[†] aspartate transaminase[‡] normal plasma liver enzyme activity for healthy BALB/c mice ^{24,25}