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RESEARCH PAPER

RAFTsomes Containing Epitope-MHC-II Complexes Mediated CD4+ T Cell Activation and Antigen-Specific Immune Responses

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ABSTRACT

Purpose To develop a liposome formulation incorporating antigen-presenting cells (APCs) membrane microdomains with enriched epitope/MHC complexes to evaluate the activities of these liposomes (RAFTsomes) to activate T cells and prime immune responses.

Methods We isolated membrane microdomain structures that contained the epitope/MHC complexes from ovalbumin (OVA) primed dendritic cells (DCs), and reconstituted them on liposomes surface by detergent dialysis. The resulted RAFTsomes were purified by density gradient centrifugation. Their T cell activation functions were evaluated by IL-2 secreting and proliferation assays *in vitro*. *In vivo* immune responses and the protective effect against OVA expressing EG.7 tumor challenge were also examined.

Results Membrane microdomains containing enriched epitope/MHC complexes can be reconstituted into liposomes with defined size and composition. The integrity and activities of these complexes after reconstitution were confirmed by *in vitro* T cell assays. OVA epitope loaded RAFTsomes injected *in vivo* resulted in high anti-OVA IgG production (predominantly IgG1). The immunized mice were protected from EG.7 tumor cell inoculation challenge.

Conclusions Based on these findings, we propose that RAFTsomes can be prepared with unique properties that may be used as an antigen delivery system for immunotherapeutic applications.

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ABBREVIATIONS

APCs	antigen-presenting cells
DCs	dendritic cells
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
MHC	major histocompatibility complex
OVA	ovalbumin

INTRODUCTION

Dendritic cells (DCs) based immunotherapies are considered to have great potential for the treatment of cancer and other diseases (1–4). The most important function of DCs is to process antigens and present them as epitope/major histocompatibility complex (MHC) complexes on their surfaces to prime T cell responses (5–7). Previous studies had explored the use of recombinant MHC molecules and their reconstitution onto liposomes (8–10). But they were found to be minimally effective for stimulating T cells without certain co-stimulation factors. Therefore Giannoni *et al.* developed a method to "precluster" MHC molecules and costimulating factors by labeling them with biotin and multiplexing with avidin on liposomes for improved activities (11).

It was reported that epitope/MHC complexes on DCs would concentrated in specialized membrane microdomains (immune synapses) with some costimulatory molecules to aid their interaction with T cells (12–15). These concentrated MHC molecules were also found on DC derived exosomes (Dex, dendritic cells exosome) which were 40-100nm naturally secreted small vesicles (16,17). The Dexs were used to prime immune responses against tumor antigens (18–20) and some preparations are under evaluation in phase I/II

clinical trials (21–23). The peptide/MHC complexes in Dexs cluster in membrane microdomains and they were considered critical for their immunological functions (24,25).

We therefore postulate that it is the membrane microdomain compositions that play important roles in retaining enriched epitope/MHC complexes for T cells activation. It may be possible for us to isolate these microdomains and test their immunological activities. Since membrane microdomains can be isolated based on the common procedure used to study lipid rafts (25–27), we developed a method to purify them and reconstitute them on liposomes to preserve the membrane protein structure and orientation. These liposomes were called "RAFTsomes". The activities of these RAFTsomes in stimulating T cells and priming immune responses were evaluated.

MATERIAL AND METHODS

Antigens and Antibodies

Ovalbumin (OVA) (grade V) was purchased from Sigma (US). $OVA_{257–264}$ (SIINFEKL) (mouse MHC-I specific epitope) and $OVA_{258–276}$ (IINFEKLTEWTSSNVMEER) (mouse I-A^b restricted epitope) were synthesed by Chengdu Kaijie Biopharm Company (Chengdu, CHN) and both were 95% pure. Mouse monoclonal [6C8] to ovalbumin was purchased from Abcam (UK). Mouse anti-MHC class II I-Ab monoclonal antibody was purchased from Millipore (Billerica, US). HRP labeled goat anti-mouse IgG(H+L) was purchased from MultiSciences Biotech (Hangzhou, CHN). HRP labeled goat anti-mouse IgG1 and goat anti-mouse IgG2a were purchased from SouthernBiotech (Alabama, US). FITC labeled mouse anti-I-A/I-E, anti-CD40, anti-CD11c and PE labeled mouse anti-I-A/I-E, anti-CD80, anti-CD86 monoclonal antibodies were purchased from eBioscience (San Diego, US).

RAFTsome Preparation

Bone-Marrow-Derived Dendritic Cell Generation and Maturation

The bone marrow-derived dendritic cells were prepared as described (28). Briefly, erythrocyte depleted bone marrow cells were obtained from C57BL/6 mice (SLAC, CHN), and cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, 10 ng/mL mGM-CSF, 1 ng/mL mIL-4 (R&D Systems, Minnesota, US). After 3 days, non-adherent cells were removed, and the medium was changed to fresh RPMI-1640 complete. Another 2 days later, the non-adherent and loosely adherent cells were harvested as immature dendritic cells (iDCs). The expression of CD11c, CD80, CD86, CD40 and MHC class II molecules on iDCs were analyzed by FACS. At day 6, half of the iDCs culture medium was changed to fresh RPMI-1640 complete medium containing 10μM OVA and 1μg/ml LPS (Sigma, US). After 24 hours, the DCs became mature DCs (mDCs) according to CD80, CD86 and MHC class II molecules expression analysis.

Purification of MHC-II Containing Lipid Rafts

To isolate the RAFT membrane fraction, 1×10^8 bone marrow derived DCs were lyzed in 500 μl MES buffer (PH 6.5) containing 1% Triton X-100 and protease inhibitor cocktail (Sigma, US) for 30min on ice. The lysates were mixed with an equal volume of 90% sucrose in MES buffer, overlaid with 5.5ml 35% sucrose and 4.5 ml of 5% sucrose in MES buffer. After centrifuged at 100,000g for 16 h, the fractions (400μl or 1 ml each) were collected from the top to bottom of the gradient. The visible band at the interface between 5% and 35% sucrose were found to contain the MHC-II lipid rafts.

Fluorometric Analysis of MHC-II Expression in Lipid Rafts

Cells were directly labeled with FITC mouse anti-I-A/I-E antibody on ice for 30min and lyzed as described above. They were spun in a sucrose gradient and harvested in 30 fractions (400 μl). A fluorometer (Hitachi F-2500, Hitachi, JP) was used to quantify the mean fluorescence intensity (MFI) in each fraction.

RAFTsome Preparation, Purification, and Characterization

DOPC and cholesterol (Avanti Polar Lipids, US) were mixed at a molar ratio of 7:3 in chloroform. After evaporating the solvent under nitrogen gas flow, the lipid film was rehydrated in PBS. Then the liposomes were extruded through 200 nm polycarbonate membrane 11 times with a mini extruder (Avanti Polar Lipids, US), and stored at 4°C. For raft reconstitution, octyl glucoside (OG) was added to the liposome solution, mixed thoroughly with purified lipid rafts at 4°C, and incubated over night. OGs were removed by dialysis against pre-cold PBS at 4°C with 6 buffer changes in 3 days. Control empty liposomes were prepared with the same procedure by using the same lipid compositions except lipid rafts. RAFTsomes were formed and then purified by ultracentrifugation for four hours at 39000rpm through a 0–40% continuous sucrose gradient. The size of purified RAFTsomes was determined by dynamic light scattering (Zetasizer Nano ZS90, Malvern, UK). The mean diameter and polydispersity index (PDI) were reported. And the transmission electron microscopy (TEM) (JEM-2010HT, JEOL, JP) morphology of the purified

RAFTsomes was observed by phosphotungstic acid (PTA) negative stain.

Western Blot Analysis

2×10^4 mature dendritic cells or lipid rafts (purified from 1×10^5 mDCs) or the RAFTsome preparation (derived from 2×10^5 mDCs) collected from sucrose density gradients were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For Western blot analysis, samples were incubated in reducing sample buffer and denatured at 95°C for 5 min. Proteins were transferred to PVDF membrane, and incubated with mouse anti MHC-II primary antibody at 4°C overnight, followed by goat anti-mouse secondary antibodies with HRP at room temperature for 2h. Enhanced Chemi luminescence (Millipore, US) was used to detect the MHC-II band.

Cell-Based Assays

IL-2 Secretion by MF2.2D9 Cells

MF2.2D9 T cell hybridoma cells, specific for I-A^b-restricted OVA peptide, was a kind gift from Prof. Xuetao Cao (The Second Military Medical University, CHN). These cells were maintained in RPMI 1640 (Invitrogen, US) supplemented with 10% FBS (PAA, AU). Each well containing 2×10^5 MF2.2D9 cells were treated for 24 h with various concentrations of RAFTsomes (w or w/o 10μM $OVA_{258–276}$) in culture medium at 37°C, 5% CO2. Supernatants were harvested and tested for IL-2 using an ELISA kit (R&D Systems, US). Results were shown as mean ±SD of triplicates.

OVA-Primed CD4+ T cell Generation and Proliferation

CD4+ T cells were collected from the spleens of OVA immunized mice. Specifically, mice were immunized by OVA (50μg)/CFA or OVA (25μg)/IFA separately by subcutaneous injection on week 0 and week 2. They were sacrificed on week 4 and spleens collected. Slpenocytes were incubated with anti-CD4 coated magnetic beads (L3T4, Miltenyi Biotec, GER) and sorted to get CD4+ T cells. Then they were suspended in RPMI 1640 containing 10% FBS at a cell density of 3×10^4/ml. Lipid rafts and RAFTsomes at different concentrations were added. After 72 hours incubation at 37°C, 5% CO_2, CCK-8 assay (Dojindo, JP) was used to quantify the number of cells. Results were shown as mean ±SD of triplicates.

Animal Studies

All the animal experiments were conducted with approval the Experimental Animal Management Committee and the Experimental Animal Ethics Committee of Shanghai Jiaotong University School of Pharmacy.

RAFTsome Immunization Studies

C57BL/6 mice were purchased from SLAC and were used between 6 and 8 wk of age. They were housed in a specific pathogen-free environment at Shanghai Jiao Tong University School of Pharmacy. For subcutaneous treatment, groups of mice (n=4) were injected on the both side of inguinal on week 0 and week 1. RAFTsomes containing different antigen doses (derived from 2×10^6, 1.2×10^7, 4×10^7 dendritic cells respectively) or OVA solutions 100ng (w/ or w/o control empty liposomes) were administered in 100 μl volume. Blood samples were taken weekly after the first immunization. Then the 2×10^6 and 1.2×10^7 groups (low dose and high dose) were reboosted with 1×10^6 EG.7 cells/100 μl /mouse 5 weeks after first RAFTsomes treatment. Blood was collected each week to monitor the OVA specific IgG level. The blood samples from 4×10^7 group were additionally used to detect both the IgG1 and IgG2a level.

EG.7 Tumor Cells Inoculation

EG.7 cells (a gift from Prof. Xuetao Cao, The Second Military Medical University, CHN) were cultured in RPMI1640 medium supplemented with 10% FBS and 400 μg/ml G418 (Sigma, US). The cells were harvested and suspended in PBS. 1×10^6 cells/mice were used to inoculate in the right flank subcutaneously in RAFTsomes treated C57BL/6 mice. The tumor size was measured with a slide caliper and tumor areas calculated using the formula Area= Width×Length. Mouse was sacrificed when the tumor size reached 300 mm^2.

OVA-Specific Antibody Detection

Anti-OVA IgG Abs in the mice sera were determined by ELISA using OVA coated plate and HRP labeled anti mouse IgG. Briefly, 96-well flat bottom plates (Corning, US) were coated with 100 μg/mL OVA in PBS at 4°C overnight, and blocked with 1% BSA in PBS at 4°C for 2 hours. Serial two fold dilutions (2^7–2^{14}) of mice sera samples were assayed in duplicate. After sera samples incubation, HRP-conjugated goat anti-mouse IgG(H+L) or IgG1 or IgG2a was added in and the reactions were developed with TMB substrate (eBioscience, US). The absorbance at 490nm was measured (Model 550, Bio-Rad, USA). For the quantitative analysis, mouse anti-OVA monoclonal IgG (6C8) was used as ELISA concentration standard.

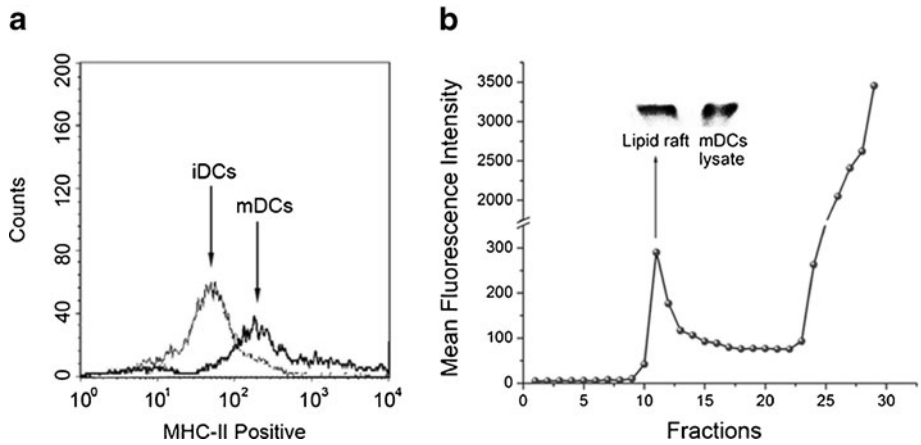

Fig. 1 The antigen pulsing of DCs and MHC-II containing lipid raft purification and characterization (representative of 3 individual experiments). (**a**) MHC-II expression after OVA&LPS stimulation detected with FACS. Thin solid line: MHC-II expression levels of immature DCs; thick solid line: MHC-II expression levels of mature DCs. (**b**) Fluorescence intensities of anti MHC-II antibody in various density gradient fractions of lysed mature DCs fractions (Insert: Western blot analysis of MHC-II from mature DCs lysate and the purified lipid raft fraction).

ELISPOT Assay

Splenocytes from individual mouse were collected by following the buffycoat separation protocol. Thin white layer was collected from the interface of the tissue homogenization and lymphocyte separation medium (Tian Jin Hao Yang Biological Manufacture CO.,LTD, CHN). The ELISPOT assay using nitrocellulose 96-well microtiter plates coated with IFN-γ (DAKEWE, CHN). Cells were added in triplicate and incubated for 24 h at 37°C in 5% CO2 in the presence or absence of 10 μM OVA protein or $OVA_{257–264}$ peptide. The numbers of OVA-specific spot-forming cell spots were developed with AEC substrate and counted using a dissecting microscope with counting software.

Statistical Analysis

All the data are given as the mean ± SD and analyzed with t-test, confidence intervals=0.95. For the tumor growth curve analysis, the growth curve was first fit to linear formulation, then compared both the slope and interval with student's T test, if both parameters were significant different in each group, then considered this two growth curves were significantly different with 0.95 confidence interval.

RESULTS

Antigen Pulsing of DCs and Isolation of MHC-II Enriched Lipid Microdomain Structures

Naive DCs were isolated from C57/BL6 mice marrow, cultured, and pulsed with the ovalbumin (OVA) antigens in the presence of LPS. The DC maturation and up-regulated MHC-II expression was monitored by FACS analysis (Fig. 1a). The MHC-II positive fluorescence signal of mature DCs (mDCs) was much higher than immature DCs (iDCs). The mDCs were lyzed, the lipid raft fragments

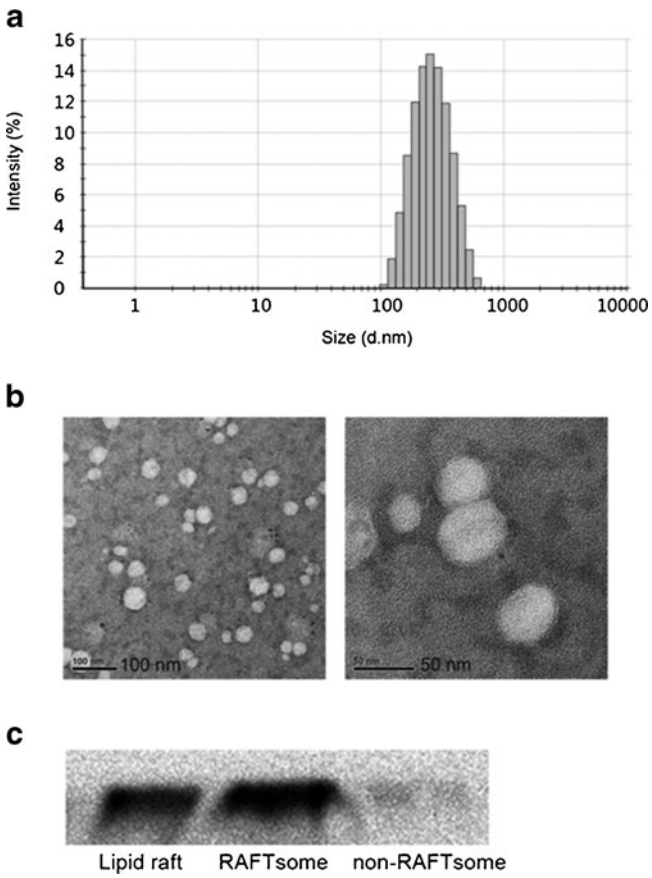

Fig. 2 Preparation and characterization of RAFTsomes (representative of more than 2 individual experiments). (**a**) Size distribution of purified RAFTsomes. (**b**) TEM micrographs of purified RAFTsomes negative stained by phosphotungstic acid. (**c**) MHC-II Western blot analysis of gradient purified lipid rafts and RAFTsomes.

were isolated and purified by sucrose gradient fractionation. Figure 1b plotted the distribution of the mean fluorescence intensities (MFI) of FITC MHC-II antibodies that were used to label the MHC-II protein on DCs before cell lysis. Totally 29 fractions were collected from the lipid raft extraction gradient from top to bottom. The fraction 11 and 12 were identified as the lipid raft fragments that were MHC-II enriched. The MHC-II presence was further confirmed by western blot analysis (Fig. 1b insert).

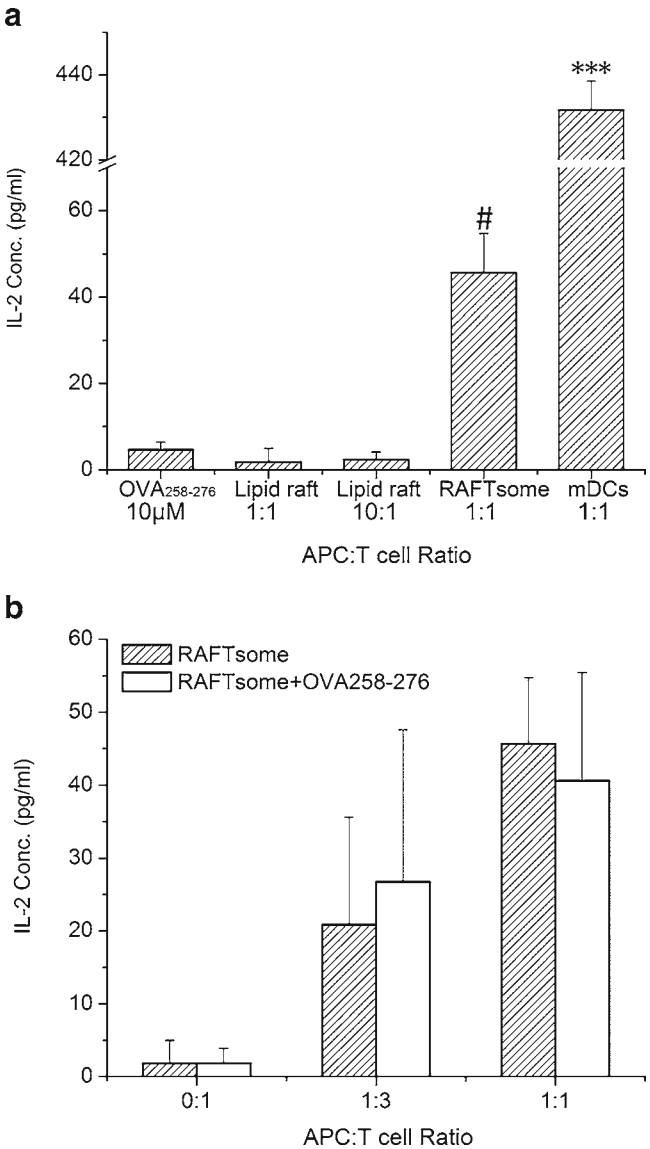

Fig. 3 RAFTsomes induced IL-2 production by OVA specific I-A^b restricted hybridoma MF2.2D9. The APC: T cell ratio was calculated as the ratio of original DCs used for preparing RAFTsomes to MF2.2D9 cells. (**a**) IL-2 secretion after RAFTsomes stimulation (n=3). (**b**) IL-2 secretion after RAFTsomes stimulation in the presence of excess epitope peptide $OVA_{258–276}$ (n=3). Results are presented as mean ± SE. The asterisk indicates significant difference ($p < 0.001$) between the marked group and each of the other groups. The pound sign indicates significant difference ($p < 0.05$) between the marked group and each of the other groups.

Reconstitution of MHC-II Enriched Lipid Rafts and RAFTsome Preparation

RAFTsomes containing the purified lipid raft fragments were prepared using a modified detergent dialysis method. Almost all the rafts could be reconstituted based on another sucrose gradient fractionation analysis. The RAFTsomes were collected at about 15% sucrose density layer, while empty liposomes were lighter at <5% sucrose density. Their size distribution and morphology were examined by Dynamic Light Scattering (DLS) (Fig. 2a) and Transmission Electron Microscopy (TEM) (Fig. 2b). They were about 200–300nm in diameter. They also contained enriched MHC-II as indicated by western blot analysis (Fig. 2c).

RAFTsomes Stimulation of OVA258-276 Specific Hybridoma MF2.2D9 Cells and IL-2 Secretion

The prepared RAFTsomes were added to $OVA_{258–276}$ specific hybridoma MF2.2D9 cells and the resulted IL-2 secretion was measured. Figure 3a showed that the RAFTsomes could significantly stimulate IL-2 production by specifically interacting with the $OVA_{258–276}$ specific T cell hybridoma. In comparison, mDCs that were pulsed with the specific antigen could induce very high IL-2 production. The RAFTsomes were less efficient at the same APC: T cell ratio. However, since the APC: T cell ratio of the RAFTsomes preparation was calculated according to the originated DC numbers used to prepare RAFTsomes, the actual

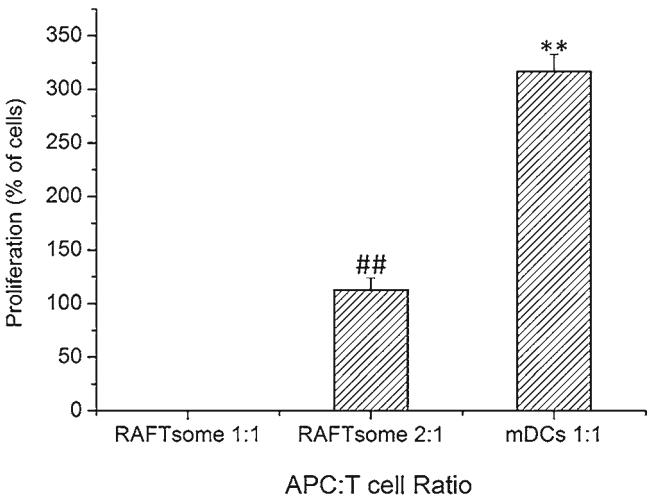

Fig. 4 RAFTsomes induced OVA primed mice CD4+ T cell proliferation. CD4+ T cells were obtained from OVA immunized mice at week 4 after immunization. The APC: T cell ratio of RAFTsomes to CD4+ T cell was calculated based on the number of original DCs used for preparing RAFTsomes (n=3). Results are presented as mean ± SE. The asterisk indicates significant difference ($p < 0.01$) between the marked group and each of the other groups. The pound sign indicates significant difference ($p < 0.01$) between the marked group and each of the other groups.

epitope/MHC-II complexes number on the RAFTsomes would be lower, because of the losses of MHCs in other membrane fractions. Surprisingly, the lipid raft fragments by themselves without the membrane reconstitution process were not able to stimulate meaningful IL-2 secretion at either APC: T cell ratios. Only after the reconstitution, RAFTsomes could stimulate MF2.2D9 T cell hybridoma in a dose dependent manner (Fig. 3b). Furthermore, the addition of more OVA epitope peptide into the RAFTsomes preparation did not result in improvement in IL-2 secretion at two different doses (Fig. 3b), suggesting the MHC-II molecules on RAFTsomes were almost fully occupied by the peptide epitopes and remained stable after the reconstitution step.

RAFTsomes Induced Proliferation of OVA-Primed Mouse CD4+ T Cells

OVA primed CD4+ T cells were obtained from the spleens of OVA immunized mice. They can interact with the OVA epitope/MHC-II complexes on RAFTsomes as well as mDCs as shown in Fig. 4. Again, the APC: T cell ratios were calculated based on the originated DC numbers used for preparing RAFTsomes. Therefore the actual epitope/

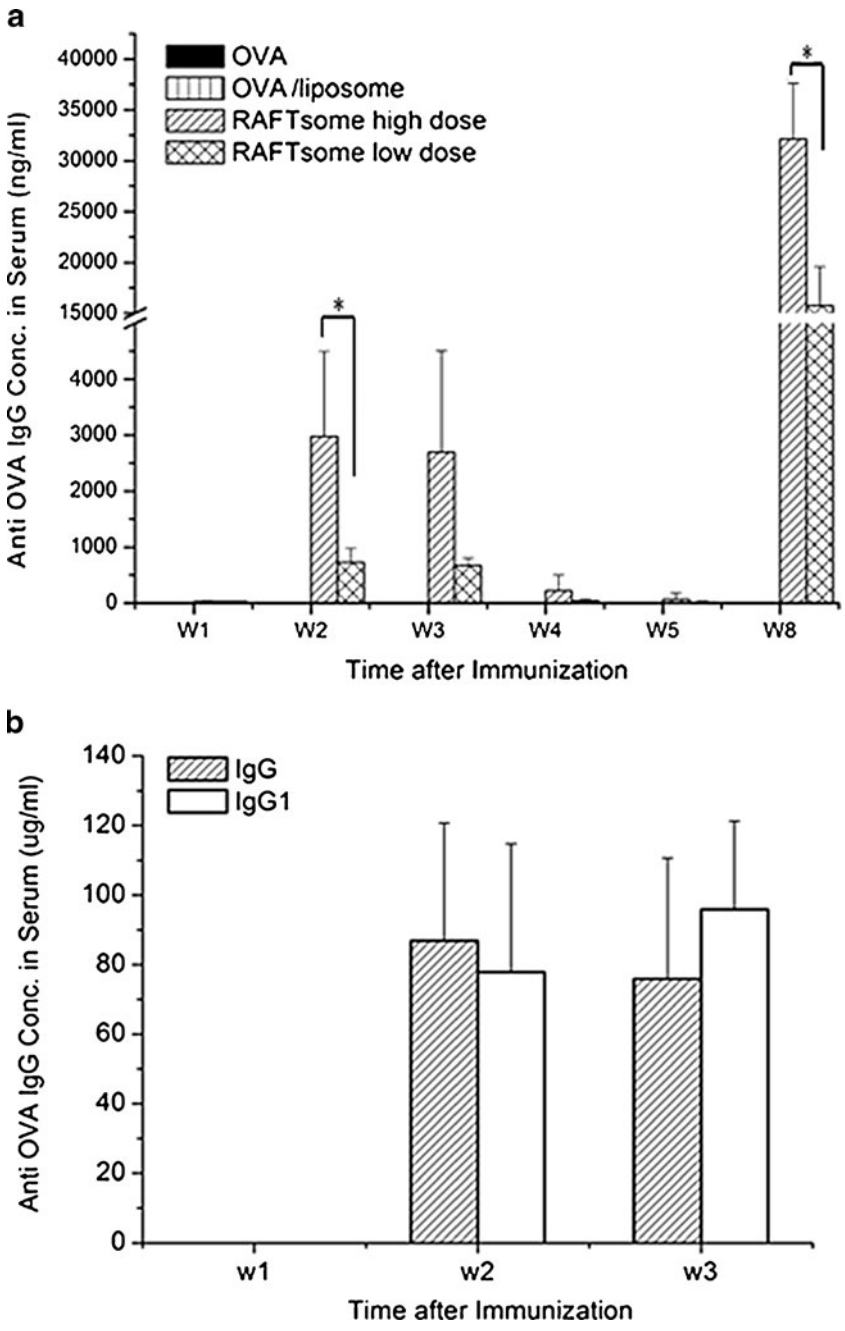

Fig. 5 RAFTsomes induced OVA specific IgG production *in vivo*. (**a**) Anti OVA IgG concentration after RAFTsomes immunization at two different dose and EG.7 cell challenge on week5 ($n=4$). Control groups were immunized by OVA (100ng) and OVA mixed with empty liposomes. (**b**) Anti-OVA IgG/IgG1 concentration with RAFTsomes immunization ($n=4$). Results are presented as mean ± SE. The asterisk indicates significant difference ($p < 0.01$) between the marked groups.

MHC-II complex numbers on RAFTsomes would be less than the corresponding mDCs.

RAFTsomes Elicited OVA-Specific Antibody Responses *In Vivo*

C57/BL6 mice were immunized at week 0 and 1 by subcutaneous injection of two different doses (2×10^6, 1.2×10^7 mDCs derived) of RAFTsomes without any adjuvant. If we assume all the MHC-II molecules that could be found in the rafts (29) were reconstituted and all loaded with epitope peptides, a rough calculation of the epitope doses in these RAFTsome preparations would suggest they were 30ng/mouse and 5ng/mouse respectively. So we also tried to immunize using excessive dose 100ng/mouse OVA antigen and antigen plus empty liposomes. There was no detectable antibody production in both groups. The RAFTsomes immunizations resulted in OVA specific antibody titers that were the highest at about 3 weeks after the prime (Fig. 5a). The titers were dose dependent and would go down after 5 weeks. The OVA specific IgG were found to be predominantly the IgG1 type (Fig. 5b). Very low IgG2a levels were detected, confirming the role of OVA epitope/MHC-II complexes in eliciting antibody responses. When the immunized mice were challenged with subcutaneous inoculation of OVA expressing EG.7 tumor cells at week5, there was a significantly boosting effect of the IgG levels in both dose groups as shown in Fig. 5a.

RAFTsome Immunization Prevented Tumor Growth after Tumor Cell Challenge

To test the immune protection effect after RAFTsomes immunization, mice were immunized with RAFTsomes twice at week0 and week1. After 6 weeks we confirmed that the OVA antibody levels in all experiment groups were back to background level to rule out the direct antibody neutralization effect. EG.7 cells were inoculated subcutaneously at a different site distant to the immunization site. The tumor nodal growth was followed and recorded in Fig. 6a. Tumors grew progressively in the saline control group that was not immunized. In contrast, almost all mice immunized with RAFTsomes did not develop tumors. In addition, we also isolated splenocytes from RAFTsomes immunized and tumor cell challenged mice. They were found to be responsive to both OVA and $OVA_{257–264}$ peptide stimulation and secret IFN-γ in the ELISPOT assay (Fig. 6b). In contrast, the splenocytes from mice without the RAFTsomes treatment hardly responded after stimulation by OVA or the epitope peptide.

DISCUSSION

Encouraged by recent success of DCs based immunotherapy (30,31), there have been significant efforts devoting to the development of new antigen presenting and immune stimulating agents that are more efficient, safer, and easier to be manufactured. Specifically, DCs derived exosomes were found to contain enriched epitope/MHC complexes and can be used to elicit tumor antigen specific immune responses (21–23). The RAFTsome preparation we made may have similar immune stimulation mechanism. They both are nano-sized lipidic particles that can interact with T cells to elicit immune responses (16,17). But since exosomes were produced by culture cells and their structures and compositions could hardly be modified, we think the RAFTsome preparation would be more versatile. In addition, they can be further modified to contain different lipid

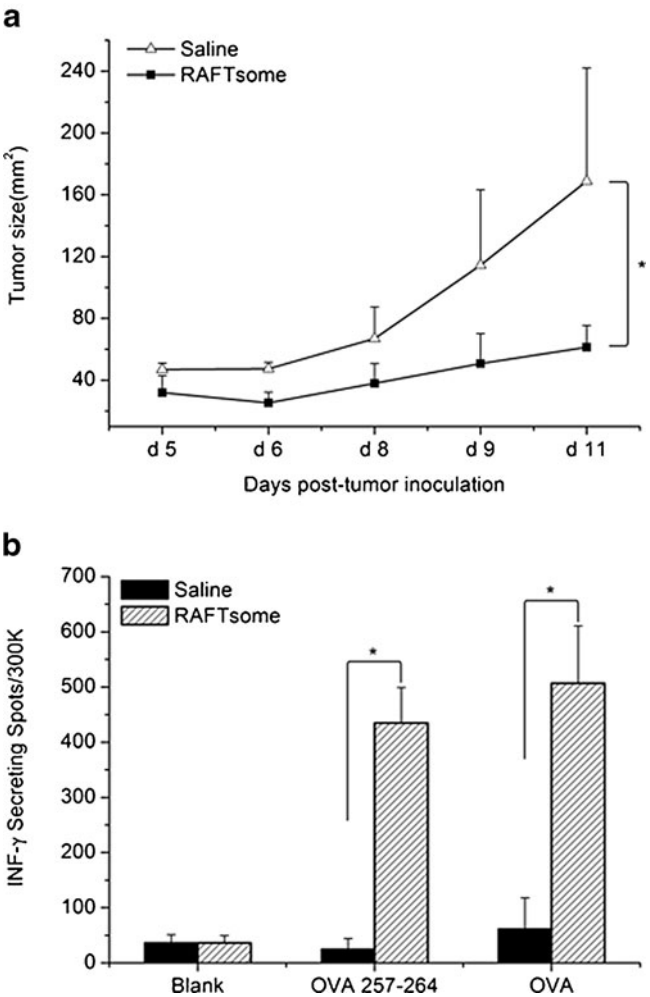

Fig. 6 EG.7 tumor challenge after RAFTsomes treatment. (**a**) Average tumor growth curves of subcutaneously implanted EG.7 tumors with (n=7) or without (n=5) RAFTsomes pretreatment. (**b**) IFN-γ ELISPOTs assay of splenocytes after EG.7 tumor cell challenge (n=5). Results are presented as mean ± SE, the asterisk means significant difference ($p<0.05$) between the marked groups.

compositions and incorporate therapeutic agents. These possibilities can be further evaluated in further studies.

In the RAFTsome preparation, we showed that the MHC-II molecules on the purified raft were mostly loaded with naturally processed epitope peptides. The proteomics analysis of the purified rafts indicated the presence of the epitope sequences (Table SI). Figure 3 demonstrated that addition of extra epitope peptides did not improve their efficacy in T cell activation. This would be advantageous compared to the exosome preparation because they required an extra step of post loading of epitopes.

The RAFTsomes after reconstitution retained significant amount of enriched epitope/MHC complexes (Fig. 2). According to Setterblad *et al.*, epitope peptide loaded MHC-II molecules were more likely to enrich inside lipid rafts (32). So we expected most of MHC-II molecules were loaded with epitopes and have higher potency than freely distributed MHC molecules (11). It is uncertain, however, whether the tightly packed lipid raft organizations were still preserved in the reconstituted liposomes. During the reconstitution process, we used octyl glucoside which actually resulted in significant reduction of particle sizes of the isolated (and aggregated) lipid rafts. So the lipid raft assembly should be at least partially disrupted. It is likely that the reconstituted liposomes only contained concentrated raft proteins without the raft structures. But on the other hand, it is also possible that because those proteins were concentrated, they may still interact with each other and had certain associated structure. Our data indicated that even though the lipid/protein assembly structures might not be the same as in cells, they retained significant immune stimulation functions.

When looking into the molecular composition of the raft fractions, we were hoping to find certain co-stimulatory factors such as CD40 which was reported to present in DCs' lipid rafts (33), and CD80 which should cluster with MHC-II (34). However, antibody staining and FACS analysis of these cofactors on the liposome surface had been difficult because of the small liposome sizes. Although preliminary proteomics analysis of the isolated rafts after peptide digestion and LC-MS/MS detection did show the presence of the OVA epitope sequences, MHC protein fragments (Table SI), as well as several raft markers such as Flotillin-1 and 2, Raftlin, and Erlin-2, other cofactors were hard to been seen because of the interference of highly abundant cytoskeleton proteins. However, the immune activity of the RAFTsomes was clearly demonstrated by functional assays for stimulating T cell activation and proliferation (Figs. 3 and 4), which implied there existed some mechanisms for co-stimulation. This is similar to what was observed with exosomes that were originated from the endo lysosomal membrane (35) and lacked the cofactor CD86 (16). Further studies are required to elucidating the detailed T cell stimulation mechanism.

In this study, we focused on CD4+ T cell mediated immune responses. The lipid rafts were extracted from mDCs which express more MHC-II and epitope/MHC-II complexes on lipid rafts (32,36). The RAFTsomes containing these complexes can act as essential TRC ligand for CD4+ T cells activation (37), which resulted in B cell differentiation and OVA specific IgG secretion. These OVA specific CD4+ T cells and B cells should persist for a long time (38), and could be reactivated by OVA or OVA expressing EG.7 tumor cells. These OVA

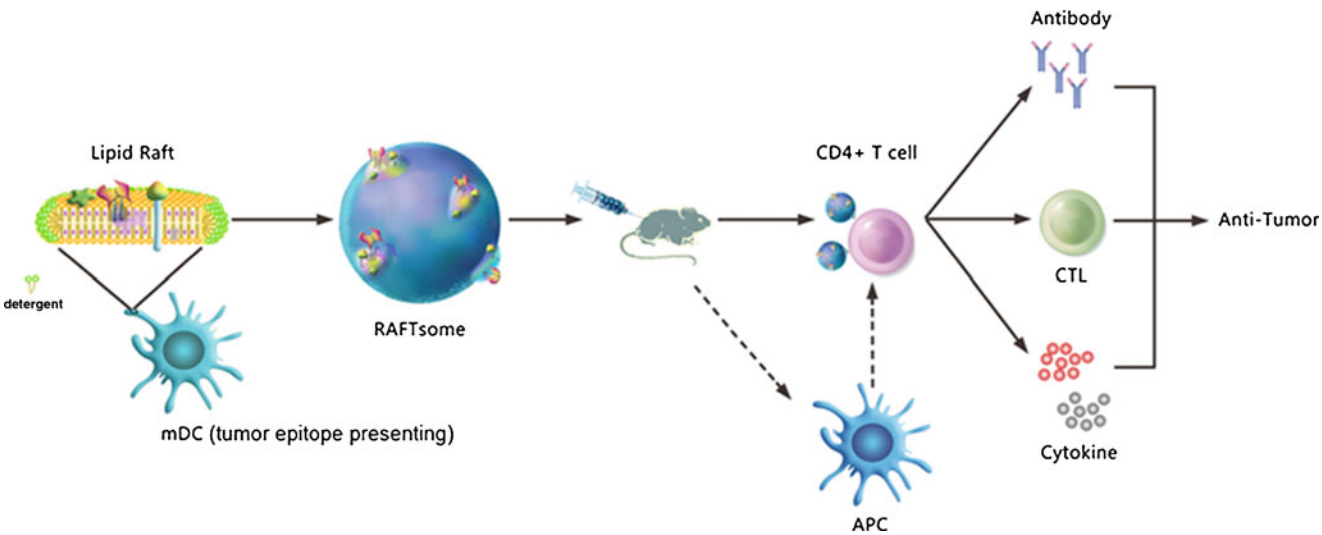

Fig. 7 Proposed schemes for RAFTsome mediated anti-tumor immune responses. RAFTsomes prepared with tumor antigen epitope/MHC-II complexes containing lipid rafts activated CD4+ T cells directly or via interaction with APCs *in vivo*. The anti-tumor effect of activated CD4+ T cells may attribute to the combined effects of tumor specific antibody neutralization; CD4+ T cells and CD8+ T cells cytotoxicity; and CD4+ T cells cytokine secretion.

specific antibodies would help to neutralize tumor cells and thereby inhibit tumor growth. We tried to summarize such an epitope/MHC-II complexes initiated mechanism in Fig. 7.

On the other hand, although some studies had suggested that MHC-I molecules were usually not found clustering in APC lipid rafts (39), the antitumor effect of RAFTsomes may involve CD8+ T cells. As many studies stated, the CD4+ T cells could help to generate and maintain CD8+ memory T cells. Cytokines such as IFN-γ produced by CD4+ T cells could also improve tumor recognition resulting cytotoxic effect on tumor cells. In addition, we think there are also possibilities for the RAFTsomes to interact with APCs *in vivo* and mediate T cells and B cells activating through epitope transfer. The epitope fragments that were presented on RAFTsome surfaces might transfer to the MHC-II molecules on professional APCs, similar as what has been suggested for exosomes (16,40). Alternatively, RAFTsomes could be taken up by DCs or other APCs which could also initiate IgG production (41) and induce cell mediated immune responses (42,43). As a result, in the challenge study by inoculating OVA expressing EG.7 tumor cells (44), we could not only observe a huge antibody response against OVA but also OVA specific T cell responses (Figs. 5a, 6b). Furthermore, there might be even some adjuvant effects resulted from the RAFTsome formulations, since lipid-based nano pariticles had been shown to have immune stimulatory activities (45,46). Liposome associated antigens could facility cross presenting to CD8+ T cell (47,48). These possibilities suggest that we can further optimize the RAFTsome formulation to improve the immunotherapeutic efficacies *in vivo*.

In summary, liposomes have been extensively studies as drug and vaccine delivery systems (49,50). RAFTsomes were prepared using well-established procedures including detergent dialysis and gradient fractionation. The membrane compositions and protein to lipid ratios could be further optimized. The size distribution of the RAFTsomes could also be controlled by membrane extrusion. So the resulted RAFTsome formulation would be more likely to meet quality control standards when entering clinical applications.

CONCLUSIONS

RAFTsomes containing the most immune reactive microdomain fragments from tumor antigen presenting dendritic cells had effective immune activity. Both *in vitro* and *in vivo* experiment confirmed the specific interaction of RAFTsomes with CD4+ T cells for efficient stimulation. In animal studies, the RAFTsomes treatment resulted specific tumor rejection effect. Our data support further studies of the development of RAFTsomes as a new antigen delivery system for immunotherapeutic applications.

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