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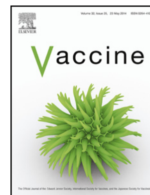

Differential immune responses to HIV-1 envelope protein induced by liposomal adjuvant formulations containing monophosphoryl lipid A with or without QS21

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ABSTRACT

Liposomes have shown promise as constituents of adjuvant formulations in vaccines to parasitic and viral diseases. A particular type of liposomal construct, referred to as Army Liposome Formulation (ALF), containing neutral and anionic saturated phospholipids, cholesterol, and monophosphoryl lipid A (MPLA), has been used as an adjuvant for many years. Here we investigated the effects of physical and chemical changes of ALF liposomes on adjuvanted immune responses to CN54 gp140, a recombinant HIV-1 envelope protein. While holding the total amounts of liposomal MPLA and the gp140 antigen constant, different liposome sizes and liposomal MPLA:phospholipid molar ratios, and the effect of adding QS21 to the liposomes were compared for inducing immune responses to the gp140. For liposomes lacking QS21, higher titers of IgG binding antibodies to gp140 were induced by small unilamellar vesicle (SUV) rather than by large multilamellar vesicle (MLV) liposomes, and the highest titers were obtained with SUV having the MPLA:phospholipid ratio of 1:5.6. ALF plus QS21 (ALFQ) liposomes induced the same maximal binding antibody titers regardless of the MPLA:phospholipid ratio. ALF MLV liposomes induced mainly IgG1 and very low IgG2a antibodies, while ALF SUV liposomes induced IgG1 ≥ IgG2a > IgG2b antibodies. Liposomes containing QS21 induced IgG1 > IgG2a > IgG2b > IgG3 antibodies. ELISPOT analysis of splenocytes from immunized mice revealed that ALF liposomes induced low levels of IFN-γ, but ALFQ induced high levels. ALF and ALFQ liposomes each induced approximately equivalent high levels of IL-4. Based on antibody subtypes and cytokine secretion, we conclude that ALF liposomes predominantly stimulate Th2, while ALFQ strongly induces both Th1 and Th2 immunity. When CN54 gp140 was adjuvanted with either ALF or ALFQ liposomes, antibodies were induced that neutralized two HIV-1 tier 1 clade C strain pseudoviruses.

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1. Introduction

The word "liposome", first coined in 1968, is a relatively nonspecific term in that it refers broadly to a hydrated vesicle having an external closed lipid bilayer and an internal volume that can be occupied by water [1,2]. Many types of liposomes containing phospholipid or nonphospholipid bilayers have been created as adjuvants for enhancing the magnitude or quality of the immune response [3–7]. For more than 35 years we have utilized a highly specialized liposome construct containing lipid A for immunization studies [3,8,9]. This construct, sometimes referred to as Walter Reed liposomes but now known as "Army Liposome Formulation" (ALF), has been used by us in various protocol formats in sixteen phase I or phase II vaccine trials [3,10–14]. The ALF liposomes have a lipid bilayer composed of phospholipids in which the hydrocarbon chains have a melting temperature in water of ≥23 °C, usually dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG). Cholesterol (Chol) is present in the

Abbreviations: ALF, army liposome formulation; ALFQ, army liposome formulation plus added QS21; Chol, cholesterol; DMPC, dimyristoyl phosphatidylcholine, DMPG, dimyristoyl phosphatidylglycerol; IFN-γ, interferon-γ; IL-4, interleukin-4; MPLA, monophosphoryl lipid A; MLV, multilamellar liposomal vesicles; NAb, neutralizing antibody; PL, phospholipids; SUV, small unilamellar liposomal vesicles.

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bilayer as a stabilizer, and monophosphoryl lipid A (MPLA) as an immunostimulator [3]. In human clinical trials the ALF-type liposomal adjuvant proved to be safe and potent in candidate vaccines to malaria, HIV-1, and cancer [3,10,13].

A similar but more complex liposomal adjuvant formulation than ALF, known as AS01 (also known as AS01B or AS01E), was introduced by GlaxoSmithKline "...for vaccines where the induction of a yet stronger T-cell-mediated immune response is required." [15]. In AS01 the lipid bilayer is comprised of a neutral lipid that is "non-crystalline" at room temperature, such as dioleoyl phosphatidylcholine; Chol; MPLA; and QS21, which is a triterpenoid glycoside saponin extracted from *Quillaja saponaria* Molina tree bark [16,17]. During manufacture of AS01 small unilamellar liposomal vesicles (SUV) are first created and purified QS21 is then added to the SUV [16,17]. The QS21 imparts unique properties in that it binds to the liposomal Chol where it causes holes or other permanent structural changes in the liposomes [18], and the reduced amount of free (nonliposomal) QS21 reduces local injection pain often caused by free QS21 [16,19,20]. The AS01 formulation is being developed as an adjuvant for a variety of vaccines [21].

In a recent study we have demonstrated that addition of QS21 to ALF liposomes (resulting in ALF plus QS21, or "ALFQ") results in complex changes in the membrane chemistry and physical structure of the liposomal membranes [22]. Factors such as MPLA:phospholipid ratio, and the relative molar concentrations and ratios of QS21, MPLA, and Chol had dramatic effects on the visibility of MPLA and Chol as determined by interactions of the binding protein in the Limulus lysate assay with MPLA and by binding of QS21 to Chol, respectively [22]. Based on the biophysical complexities of ALF and ALFQ, we have hypothesized that distinctive differences in the physical structures might affect the abilities of these formulations to serve as vaccine adjuvants.

The goal of this study was to explore the relative effects of seven different compositions of ALF-type formulations on the induction of immunity to CN54 gp140 antigen. The CN54 gp140 protein is useful as a model antigen in that it is a commercially available HIV-1 clade C trimeric envelope protein that has been used in human vaccine trials [23], and has been studied as a model antigen with various adjuvants [24–28]. Here we focused on the effects of different compositions of large multilamellar vesicle (MLV) liposomes and small unilamellar vesicle (SUV) liposomes, with or without QS21, on the induction of binding antibodies, IgG subtypes, IL-4 and IFN-γ production, and the induction of neutralizing antibodies to HIV-1 clade C.

2. Materials and Methods

2.1. Materials and reagents

DMPC, DMPG, and synthetic MPLA (PHAD^{TM}), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DMPC and Chol were dissolved in freshly distilled chloroform, and DMPG and MPLA were dissolved in chloroform:methanol (9:1). Purified QS21 (Desert King International San Diego, CA, USA) was dissolved in PBS at 1 mg/ml. Horseradish peroxidase (HRP)-linked-sheep anti-mouse IgG was purchased from The Binding Site (San Diego, CA, USA (cat. no. AP272). Goat anti-mouse IgG1 (A90-105P), IgG2a (A90-107P), IgG2b (A90-109P), IgG3 (A90-111P), all linked to HRP, and purified mouse IgG1 (MI10-102), IgG2a (MI10-103), IgG2b (MI10-104), and IgG3 (MI10-105) were purchased from Bethyl Laboratories (Montgomery, TX, USA). Unconjugated goat anti-mouse IgG Fab (1015-01) was from Southern Biotech (Birmingham, AL, USA). Purified anti-mouse interferon-γ (IFN-γ) (51-2525KZ), purified rat anti-mouse interleukin-4 (IL-4) (BVD4-1D11), biotin-labeled anti-mouse IFN-γ (51-1818KA), and biotin-labeled rat anti-mouse IL-4 (BVD6-24G2) were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

2.2. Culture of splenocytes

Dulbecco's modified Eagle's medium (DMEM) (from Quality Biological Inc., Gaithersburg, MD USA) containing 10% fetal bovine serum (FBS) (from Gemini Bioproducts, Woodland, CA USA), and 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine were used for culturing TZM-bl cells and for HIV-1 neutralization assays. Complete RPMI 1640 medium (cRPMI) containing 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine, were used for culturing mouse splenic lymphocytes and for ELISPOT analyses.

2.3. Preparation of liposomes and vaccines

Formulations containing DMPC, DMPG, Chol, and MPLA (ALF liposomes) were prepared by the lipid deposition method previously described [14]. Lipids were mixed and dried by rotary evaporation. MLV were formed by adding PBS, pH 7.4, at a final concentration of either 50 mM, or 20 mM, or 1.272 mM of total phospholipids, respectively, relative to the volume of water in the aqueous liposome suspension. A constant amount of MPLA (0.227 mM) relative to the aqueous volume was also present in each liposome preparation, resulting in molar ratios of MPLA to phospholipids of 1:220, 1:88, and 1:5.6, respectively. The epitope density (mol%) of MPLA in the lipid bilayer increased proportionally to the decreased amounts of phospholipid and Chol (and QS21, when present) in the bilayer (Table 1). Liposomal DMPC, DMPG, and Chol were in molar ratios relative to each other of 9:1:7.5, resulting in a liposomal Chol concentration of 43 mol% relative to the phospholipids in all ALF preparations lacking QS21. Army liposome formulations plus QS21 (ALFQ) were made by mixing QS21 with MLV or SUV liposomes in which the liposomal Chol concentration was 55 mol% relative to the total phospholipid. The QS21 irreversibly binds to the Chol in the liposomes under these conditions with no detectable free QS21 present in the buffer [22].

Table 1 summarizes the chemical composition of each of the seven liposomal formulations. The table also shows some of the physical characteristics of each preparation as determined with a Malvern Zetasizer Nano ZSP (Malvern Instruments, Inc., Westborough, MA, USA). Light microscopy of ALF MLF and ALFQ was performed with an Olympus BH-2-RFCA microscope at 500× magnification with an Olympus DP71 camera. Liposomes were microfluidized using a Microfluidics LV1 low volume high shear microfluidizer (Microfluidics, Westwood, MA, USA) at 30,000 psi to form SUVs. The diameter size distributions of liposomes were measured by Horiba LB-550 particle sizer (Horiba Scientific, New Jersey, NJ, USA). The total amount of QS21 in each ALFQ formulation used for immunization was 10 μg, the amount of MPLA was 20 μg, and the injection volume of each ALF or ALFQ preparation was 50 μl.

A total injection dose of 10 μg of HIV-1 clade C gp140 CN54 envelope antigen (purchased from Polymun Scientific Inc., Klosterneuberg, Austria) was mixed with each formulation ALF or ALFQ liposomes in PBS. Four different ALF plus gp140 formulations were made, having liposomal MPLA:phospholipid ratios of 1:220 or 1:88 for MLV, and 1:88 or 1:5.6 for SUV. Three different ALFQ plus gp140 formulations were prepared by adding QS21 to ALF SUV or ALF MLV, with the ALF MLV having MPLA:phospholipid ratios of either 1:220 or 1:88 and the ALF SUV 1:5.6.

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Table 1
Physicochemical properties of liposomes used in the immunizations.

Liposomes		MPLA: phospholipid (molar ratio)	Chol (mol%)[a]	Amount of liposomal lipids per injection dose (50 μl)					MPLA (mol%)[b]	QS21 (mol%)[b]	*Z*-average (nm)	Poly-dispersity index	Zeta potential (mV)
				DMPC (nmol)	DMPG (nmol)	Chol (nmol)	MPLA (nmol)	QS21 (nmol)					
MLV	−QS21	1:220	43%	2250	250	1886	11.35	None	0.26	None	6358	1	−15.07
MLV		1:88		900	100	754			0.65		6266	0.970	−13.84
SUV		1:88		900	100	754			0.65		59.38	0.370	−12.36
SUV		1:5.6		57.2	6.3	48			10.17		80.90	0.181	−18.47
MLV	+QS21	1:220	55%	2250	250	3055		5.025	0.2	0.09	5952	0.739	−13.96
SUV		1:88		900	100	1222			0.51	0.22	2506	1	−14.77
SUV		1:5.6		57.2	6.3	77.7			7.75	3.29	2130	1	−16.84

[a] Mol% of Chol was calculated with respect only to phospholipids and cholesterol in the lipid membrane.
[b] Mol% of MPLA or QS21, respectively, was calculated with respect to all constituents in the lipid membrane.

2.4. Cholesterol analysis

Cholesterol content was analyzed to confirm the Chol, and indirectly the phospholipid concentration as described previously [22,29].

2.5. Immunization of mice

Female BALB/c mice (Charles River Laboratories, Indianapolis, IN, USA) (5–6 weeks of age; 6/group) were immunized IM with 0.05 ml of the vaccines by injection in alternate rear thighs at 0, 3, and 6 weeks. Four mouse groups were immunized with the ALF plus gp140 formulations listed above and three groups were immunized with the ALFQ plus gp140 formulations. One mouse group was not immunized and was used as negative control for the immunological studies. The animals were bled prior to the first immunization and 3 and 6 weeks after the primary immunization. At week 9, the animals were terminally bled and the spleens were collected.

2.6. ELISA

To determine the endpoint titers of IgG antibodies, gp140 protein (0.1 μg/0.1 ml/well in PBS) was added to Immulon 2HB flat bottom plates (Thermo Fisher Scientific, Waltham, MA, USA). After incubating overnight at 4 °C all further steps were performed at RT. Plates were blocked with 200 μl of 0.5% milk/0.1% Tween 20 in PBS (blocking buffer) for 2 h. Samples were serially diluted two fold starting with 1:400 dilution, and 100 μl of diluted serum samples were added in triplicate to the plate. Plates were incubated for 1 h and washed 4 times with TBS/0.1% Tween 20. HRP-linked sheep anti-mouse IgG (0.1 μg in 100 μl blocking buffer) was added to each well and plates were incubated for 1 h followed by washing. One hundred microliters of ABTS 2-component substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) were added to each well, and plates were incubated for 1 h. Color development was stopped by adding 100 μl/well of 1% SDS. The absorbance was read at 405 nm. End point titer is defined as the dilution at which the absorbance was twice background.

IgG subtype determination was modified from Glenn et al. [30]. A standard curve was made by using unconjugated goat anti-mouse IgG (Fab). One hundred microliters of goat anti-mouse IgG Fab (1 μg/ml in PBS) was added to the wells of 96-well Immulon 2HB flat bottom plates and the plates were incubated overnight at 4 °C. Plates were washed 3 times with 0.05% Tween 20 in PBS (washing buffer), blocked with 250 μl/well of 0.5% skim milk in PBS (blocking buffer), and incubated at RT for 1 h. After washing, 100 μl of blocking buffer were added to each well. Purified mouse IgG1, IgG2a, IgG2b, and IgG3 were diluted two-fold starting from 200 ng/ml of blocking buffer. One hundred microliters of each dilution were added to the plates. Plates were incubated at RT for 2 h, and washed 3 times. One hundred microliters of HRP-linked goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (1:1000 dilution in blocking buffer) were added to the corresponding wells, and plates were incubated at RT for 1 h. Plates were washed and 100 μl of ABTS were added. Color development was stopped by adding 100 μl/well of 1% SDS. Absorbance was read at 405 nm. For subclass analysis, individual mouse serum in each group was added to gp140-coated plates as for the standard ELISA. HRP-linked goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 were added and the ELISA was conducted together with the standard curve. The concentration of the immunoglobulin subclasses of the individual sera of each group was calculated using the standard curve.

2.7. Enzyme-linked ImmunoSpot (ELISPOT) assays for IFN-γ and IL-4

Spleens from euthanized mice, were pressed through a 100-μm nylon cell strainer (Thomas Scientific, Swedesboro, NJ, USA, cat No. 4620F05) with the plunger of a syringe. Splenic cell suspensions were collected in and washed 3 times with cRPMI medium. ELISPOT was conducted as described previously [31]. Prior to the harvesting of spleens, multiScreen 96-well microtiter plates (EMD Millipore, Billerica, MA, USA) were pre-treated with 70% ethanol, washed 3 times with PBS, and coated with 100 μl/well of either 5 μg/ml of capture IFN-γ-specific IgG diluted in PBS or 2 μg/ml of capture IL-4-specific specific IgG diluted in PBS. Plates were incubated overnight at 4 °C, then washed 2 times with cRPMI and blocked with 200 μl/well cRMPI at RT for 2 h. Fifty microliters of mouse cell suspensions (8×10^6 cells/ml) were plated in duplicate for each cytokine assay. Either 50 μl of cRPMI for unstimulated cells, or 50 μl of 10 μg/ml gp140 CN54 for stimulated cells, or with 50 μl of 10 μg/ml Con-A as a positive control, or 50 μl of 2 μg/ml PHA as negative control were added to corresponding wells. Plates were incubated for 18 h at 37 °C in a CO_2 incubator. Plates were washed 3 times with PBS containing 0.002% Tween 20 (washing buffer). One hundred microliters of 2 μg/ml biotin-labeled detection IFN-γ or IL-4 antibodies in PBS were added to each well. Plates were incubated at RT for 3 h and then washed 3 times. One hundred microliters of streptavidin-alkaline phosphatase solution (Southern Biotech, cat. No. 7100-04) diluted 1:1000 in PBS/5%FBS/0.001%Tween) were added to each well, and plates were incubated at room temperature for 1 h in dark. Plates were washed 3 times and 100 μl/well of BCIP/NBT chromogen substrate (Kirkegaard & Perry, cat. No. 50-81-07) were added. After spots developed, plates were thoroughly rinsed with distilled water. The frequencies of IFN-γ and IL-4-producing cells were determined with a BioReader 3000 Elispot Reader (Bio-Sys GmbH, Karben, Germany). Data are expressed as the mean number of spots.

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2.8. Neutralization assay

Neutralizing antibodies were measured with two tier 1 HIV-1 Env pseudoviruses by using luciferase-based virus neutralization assays with TZM-bl cells as previously described [32]. The 50% inhibitory dilution of serum (ID_{50}) was calculated as the serum dilution that resulted in a 50% reduction in relative luminescence units compared with the level of virus in wells containing pre-immunization serum after the subtraction of cell control relative luminescence units. Briefly, 4-fold serial dilutions of serum samples in 25 µl were assayed in a 96-well flat-bottom plate in duplicate in 10% DMEM. HIV clade C, GS015 pseudovirus and MW965.26 pseudovirus (25 µl) were added to each well and the plates were incubated for 1 h at 37 °C. TZM-bl cells were then added (10^4 cells/well) in a 50 µl volume in 10% DMEM containing DEAE-dextran and neutralizing antibody titers were determined after 48 h [32]. Murine leukemia virus (MuLV) negative controls were included in all assays.

2.9. Statistical analysis

The entire experimental immunization protocol was performed independently twice, with each immunization experiment having identical groups of 7 adjuvant formulations, in which each of the groups had 6 mice. In one of the two independent experiments the ALFQ MLV group with MPLA:phospholipid ratio of 1:220 suffered an inadvertent loss of 4 of the 6 mice and the data of that group are based on 2 mice. Statistical analysis was performed using GraphPad Prism for the data of each independent experiment. For antibody subtype quantification, sigmoidal 4 parameter nonlinear regression curve fitting was used. Statistical comparison between multiple groups was performed using one-way ANOVA, Kruskal–Wallis test with Dunn's correction. Column comparison analyses were performed using unpaired *t*-test (Mann Whitney test).

3. Results

3.1. Physicochemical characteristics of ALF and ALFQ liposomes

To test the effects of liposome size on adjuvant potency, large MLV and SUV, each being a type of ALF liposomes (i.e., containing MPLA), were constructed. As shown in Fig. 1A, based on light scattering analysis the diameter size range of the ALF SUV particles was between 50 and 100 nm, and ALF MLV was between 1 and 4 µm. Even at a high MPLA:phospholipid ratio ALF SUV formed a single distribution of small particles. However, in contrast to liposomes lacking QS21, the size of all ALF liposomes containing QS21 (ALFQ), regardless of whether the initial ALF particles were SUV or MLV, were measured in the micrometer range (Fig. 1B). In each case, each measured particle population was narrow and had only one peak. Within the overall size range of ALFQ particles distinct individual subpopulations of large ALFQ having slightly different sizes occurred depending on the MPLA:phospholipid ratio (Fig. 1B).

Although these results with MLV ALF particles, and with ALFQ, containing QS21 indicate that light scattering analysis detected many large particles, it has been shown previously that MLV liposomes also contain numerous small (submicron) particles in a hyperbolic size distribution as determined by electronic particle size analysis [33]. Thus, with light scattering analysis the small particles that were present in the MLV were overshadowed and obscured by the large particles. The large differences in the sizes of ALF SUV compared to ALF MLV and to all of the ALFQ particles are reflected in the *Z*-average sizes (Table 1). The broad size distribution was also responsible for the relatively large polydispersity indexes (>0.7) of all of the liposomes except for the ALF SUV (Table 1). Fig. 1C illustrates that a wide range of size distribution of large particles was visible by light microscopy, both in ALF MLV, and in ALFQ created by adding QS21 either to ALF SUV or ALF MLV.

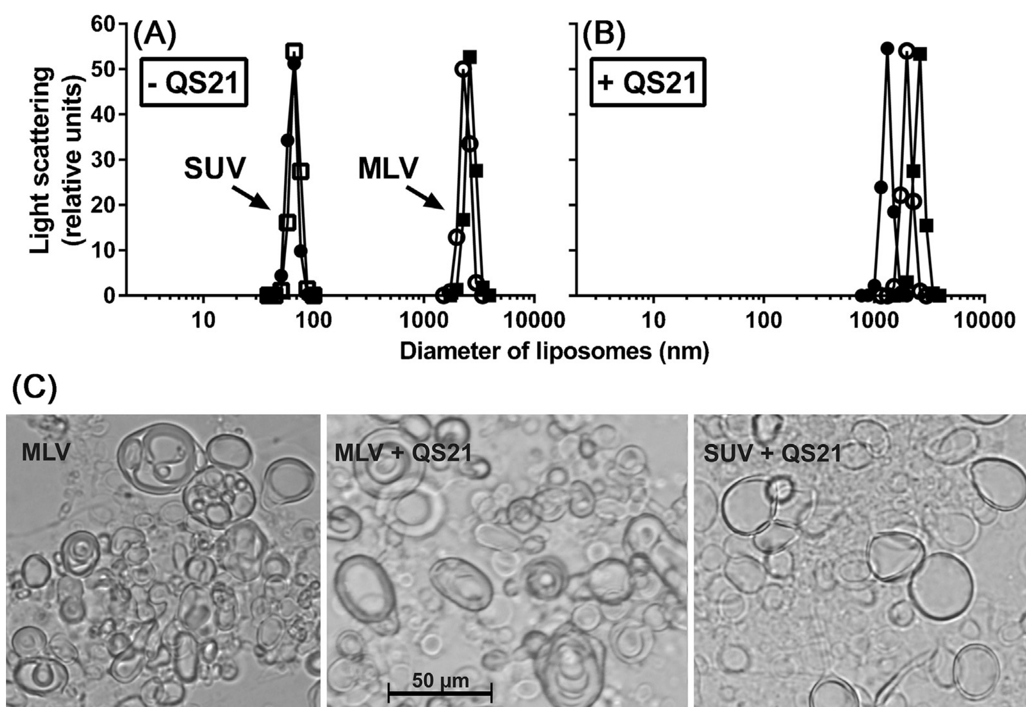

Fig. 1. Size distributions as measured by light scattering of ALF SUV and ALF MLV in the absence (A) and presence (B) of QS21. (A) The two SUV curves comprised liposomes having MPLA:phospholipid ratios of 1:88 or 1:5.6. The two MLV curves comprised (from left to right): liposomes having MPLA:phospholipid ratios of 1:88 or 1:220. (B) The curves represent ALFQ in which the initial ALF to which QS21 was added comprised (from left to right): SUV with MPLA:phospholipid ratios of 1:5.6 and 1:88, and MLV with MPLA:phospholipid ratio of 1:220, respectively. (C) Light microscopy (1:500×) (left to right).of ALF MLV (MPLA:PL ratio 1:220), ALFQ MLV (MPLA:PL ratio 1:220), ALFQ SUV (MPLA:PL ratio 1:88).

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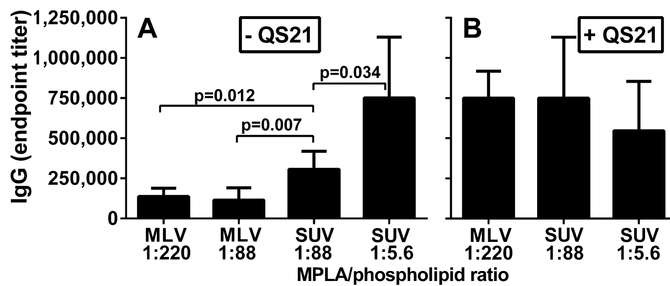

Fig. 2. IgG endpoint titers to gp140. Mice were immunized with ALF (−QS21) + gp140 (A) and ALFQ (+QS21) + gp140 (B) containing the indicated MPLA:phospholipid molar ratios in which 20 μg of MPLA, and where indicated 20 μg of QS21, were present in each formulation. Sera were from week 9 and were assayed by ELISA. Values are the mean of 6 animals/group ± SD.

Interestingly, by light microscopy it appears that the large particles in ALFQ created from SUV ALF were composed mainly of large unilamellar vesicles (LUV) (or possibly oligolamellar vesicles) and those created from MLV Alf were composed mainly of large MLV with fewer, if any, LUV (Fig. 1C). Because of the wide size distributions of ALF MLV and ALFQ the relative effects caused by different liposomal surface charges (as reflected by zeta potential in Table 1) is difficult to interpret. However, it is noted that when they were made with MPLA:PL ratios of 1.5.6, the ALF SUV and ALFQ had the strongest negative charges, presumably due to the greater negative charge provided by the increased epitope density (mol%) of MPLA (Table 1).

3.2. Binding antibodies to gp140 induced by using ALF SUV, ALF MLV, or ALFQ liposomes

As shown in Fig. 2A, ALF SUV containing MPLA:phospholipid in the ratio of 1:88 induced significantly higher IgG anti-gp140 binding antibodies in mice than ALF MLV having the same MPLA:phospholipid ratio. ALF SUV with an MPLA:phospholipid ratio of 1:5.6 induced the highest titer of binding antibodies. Fig. 2B shows that all of the ALFQ-adjuvanted formulations induced higher binding antibodies to gp140 than any of the ALF MLV-adjuvanted formulations (Fig. 2A). The total gp140-specific IgG binding antibody levels in all ALFQ-adjuvanted groups was independent of the original SUV or MLV to which QS21 was added, or of the relative phospholipid concentration. The titration curves of each of the individual mice in Fig. 1 are shown in Supplemental Fig. 1. The binding antibody titers for individual mice in the second immunization are shown in Supplemental Fig. 2.

3.3. IgG subclasses

All mouse groups immunized with gp140 together with ALF had moderate or high titers of IgG1 binding antibodies to gp140 (Fig. 3A). ALF MLV induced predominantly IgG1 subclass, while ALF SUV induced a balance between IgG1 and IgG2a production and also induced IgG2b production. Increasing the MPLA:phospholipid ratio of ALF SUV from 1:88 to 1:5.6 resulted in higher antibody responses of the subclasses (Fig. 3A). As shown in Fig. 3B, in the ALFQ groups the serum concentration of IgG1 was consistently high; and IgG2a, IgG2b, and even a small amount of IgG3 subtypes were induced, but the relative levels of subtypes were independent of the MPLA:phospholipid ratios of ALF SUV or ALF MLV initially utilized for creation of ALFQ (Fig. 3B). As shown in Fig. 3C, based on the IgG1/IgG2a ratios the ALF MLV in general appeared to have a relatively strong Th2 bias when compared to the more balanced Th1/Th2 levels of ALF SUV and ALFQ.

3.4. Antigen-specific IFN-γ and IL-4 production

As shown in Fig. 4, all of the ALF-adjuvanted formulations induced numbers of IFN-γ-producing splenic lymphocytes that were higher than those observed with naïve control splenocytes. ALFQ formulations induced significantly higher numbers of IFN-γ-positive cells than ALF (Fig. 4A). All of the immunized mice exhibited high IL-4 production that was significantly higher than naïve mice ($p < 0.0001$) (Fig. 4B). Significant differences between IL-4 producing cell were not observed among the various immunized groups.

3.5. Neutralization of HIV-1

Because neutralizing antibodies (NAbs) are widely viewed as being important for a prophylactic vaccine to HIV-1 we examined the ability of ALF and ALFQ formulations to induce NAbs to the model gp140 envelope protein. Pooled serum samples from each of the 7 immunized mouse groups in each of the two independent experiments were tested against two tier 1, clade C HIV primary isolates. As shown in Fig. 5, in one of the two experiments one ALF-adjuvanted and two ALFQ-adjuvanted vaccine groups induced anti-gp140 antisera that neutralized both MW965.26 pseudovirus (Fig. 5A) and GS015 pseudovirus (Fig. 5B) at high ID_{50}. ALF having an MPLA:phospholipid ratio of 1:5.6 induced the highest neutralization titer, while ALFQ with 1:88 and 1:220 MPLA:phospholipid ratios also induced NAbs, but with somewhat lower titers. In the second of the two experiments higher ID_{50} neutralization levels were observed and all of the groups induced NAbs.

4. Discussion

This work represents a functional analysis of adjuvant activities that is based on parallel observations from our previous report which described the structural characteristics of ALF-type liposomes containing MPLA with or without addition of QS21 [22]. However, as noted in a recent review, “Vaccine adjuvants are defined by what they do, not by what they are.” [6]. Here we demonstrate that the detailed membrane composition and physical biochemistry of different liposomes strongly defines the adjuvant characteristics of individual formulations as determined by quantitative and qualitative immune responses to a model antigen consisting of CN54 gp140 HIV-1 envelope protein. In this work each of the immunized animals in each of two identical experiments received the same dose of antigen and liposomal MPLA, and therefore the only major variables were the physical and chemical characteristics of the liposomes and liposomal lipids. The four main liposomal variables examined were particle size, number of lipid bilayers (unilamellar vs multilamellar), liposomal MPLA:phospholipid ratio, and the presence or absence of QS21.

With respect to particle size, ALF SUV liposomes were found to be homogeneous nanoparticles between 50 and 100 nm in diameter, while ALF MLV liposomes contained numerous large particles with diameters between 1 and 4 μm. However, addition of QS21 to ALF SUV had a profound effect on the particle size in that the light scattering properties of the resultant ALFQ particles, regardless of whether they were created by adding QS21 to SUV or to MLV, were unexpectedly similar to those of the large ALF MLV liposomes. The reason that QS21 added to ALF SUV caused an increased number of large size particles is not yet known, but it might have been either through changes in the size or structure of the liposomes themselves, Perhaps it occurred because of the presence of the 55% Chol that was used in ALFQ that caused, as shown in Table 1, a greater epitope density of QS21 which is a relatively large glycolipid that would increase the surface area and volume of the lipid bilayer by

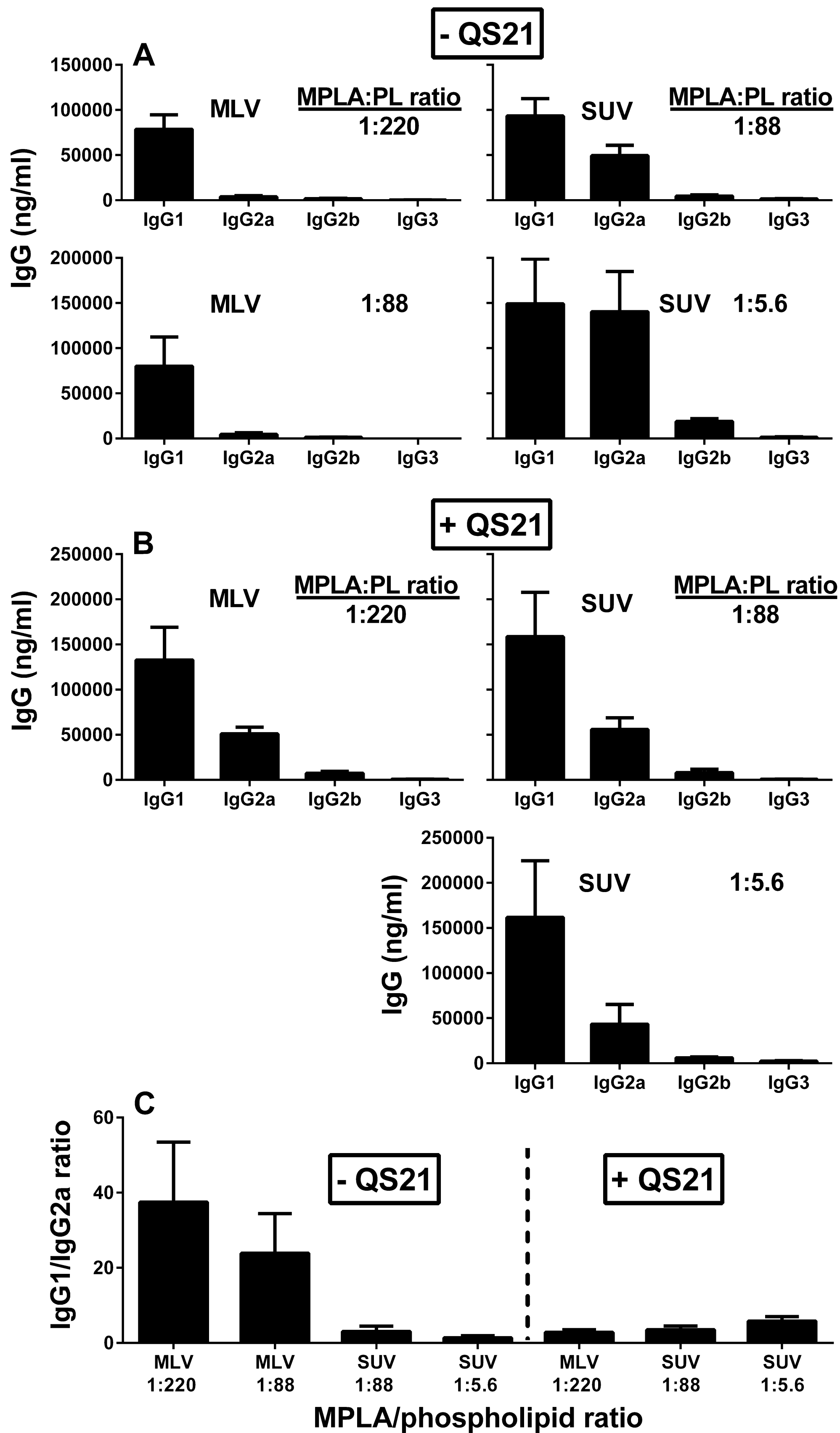

Fig. 3. IgG subtype profiles of anti-gp140 antisera. Mice were immunized with ALF + gp140 (A) or ALFQ + gp140 (B). ELISA plates were coated with gp140. Subtype analyses were conducted and values were calculated from standard curves. Values are from week 9 of immunized mice and are the mean of 6 animals/group ± SEM. (C) IgG1/IgG2a ratios.

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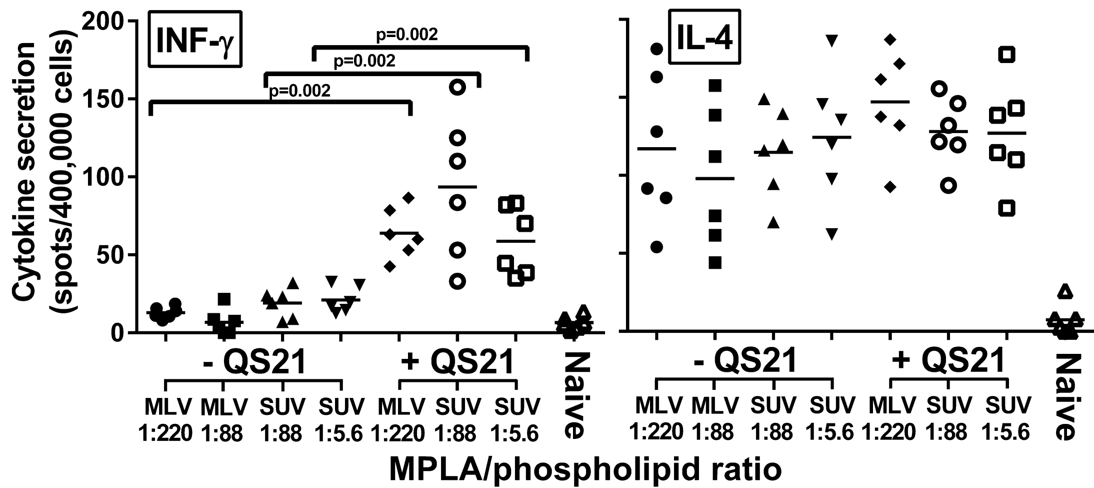

Fig. 4. Production of IFN-γ and IL-4 by splenocytes from individual mice 9 weeks after primary immunization. Splenocytes were plated and stimulated with gp140 for 18 h and the number of cytokine producing cells were determined by ELISPOT. The naïve controls represented mice who were not immunized.

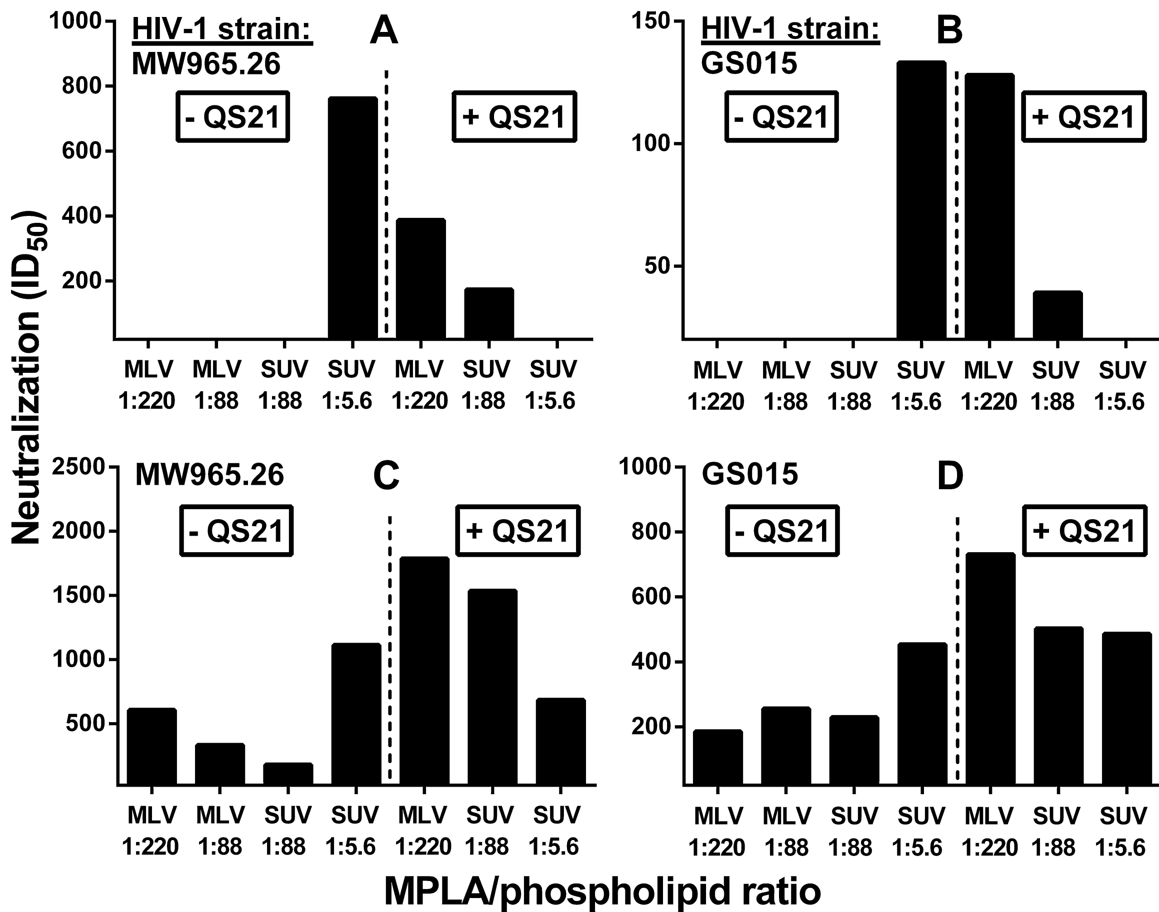

Fig. 5. Neutralization of HIV by pooled antisera. The vaccine formulation is indicated on the *x*-axis. ID50 values represent 50% neutralization of pseudoviruses MW965.26 pseudovirus (A, C) and GS015 pseudovirus (B, D) at the indicated dilution of the serum samples from mice 9 weeks after primary immunization. The naïve controls were pooled sera from non-immunized mice. ID50 values <20 were considered as background. MuLV pseudovirus was used as an assay negative control (data not shown). (C) and (D) are data for identical repeat experiments of (A) and (B).

binding to Chol. Alternatively, it occurred because of aggregation of liposomes, or because of novel structures caused by the presence of QS21. Based on light microscopy (Fig. 1C), any of these possibilities might have been responsible for some or all of the large sizes of ALFQ. If novel structures did occur the resultant particles still had round vesicular shapes. However, we did note that the microscopic appearance of ALFQ made by adding QS21 to SUV appeared to have a large number of large uni- or oligolamellar vesicles. This suggests that the addition of QS21 to SUV might simply have made the SUV grow to a larger size. In contrast, ALFQ made by adding QS21 to MLV had a large number of multilamellar vesicles and fewer, if any, large unilamellar vesicles. It is noted that the commercial AS01 adjuvant formulation is manufactured in a described manner similar to that of ALFQ by adding QS21 to SUV liposomes containing

both Chol and MPL™ [17]. In view of this, although the sizes of the commercial AS01 particles have not been disclosed, it is possible that they might contain a population of large uni- or oligolamellar particles.

In the case of ALF-type liposomes that lacked QS21, high titers of IgG binding antibody titers were induced by large MLV liposomes, but the titers were increased more than two-fold by using the small (SUV) liposomes. Increased adjuvant potency of small when compared to large liposomes has been observed previously with different types of liposomes and this has led to a number of hypothetical explanations [4,34]. In the present instance one likely simple explanation is that the MPLA may be largely present on the inner bilayer membranes of MLVs when compared with the same number of MPLA molecules present in SUV liposomes and may be less visible to the immune system. Although the reason for the immunological differences found based on size is not yet clear, it is also possible that in the absence of QS21 ALF SUV has greater access than ALF MLV to direct drainage to lymph nodes, as opposed to cellular transport of MPLA, as suggested by studies with subcutaneous injection of nanoparticle antigen [35]. However, in the case of ALFQ liposomes, all of which had large sizes equivalent to those of the ALF MLV liposomes, a significantly greater tendency to a Th1 type response was observed as determined IFN-γ secretion. Thus, with ALFQ liposomes the Th1 responses were related more to the known Th1 tendencies associated with QS21 rather than to the initial sizes of the liposomal particles.

Perhaps the most surprising observation in this study was that increasing the MPLA:phospholipid ratio of ALF SUV from 1:88 to 1:5.6 had dramatic effects not only on the magnitude of the immune response (Fig. 2A), but also on the induction of IgG2a subtype antibodies (Fig. 3A) and on the induction of NAbs (Fig. 5). In considering the cause of this unexpected result we note that the number of phospholipid molecules surrounding the MPLA in the lipid bilayer is greatly reduced at an MPLA:phospholipid molar ratio of 1:5.6 when compared to ratios of 1:88 or 1:220. The net result in the present context could have been that the phosphorylated diglucosamine polar headgroup of MPLA, which is presumably mainly responsible for the adjuvant activity of MPLA, was in a higher epitope density (mol% of MPLA, as shown in Table 1) on the liposome surface and was less shielded by the phosphocholine headgroups of the surrounding lipid bilayer, thus making the MPLA headgroup potentially more visible as a danger signal [36]. However, it is also known that lipid A can form nonlamellar cubic and inverted hexagonal phase II structures under certain conditions [37]. If structures such as these occurred at a high MPLA:phospholipid ratio in the liposomes they would have represented dramatic changes in the three dimensional molecular architecture of the molecular aggregates, and direct interactions of these structures with gp140 might have provided opportunities for changes in the conformational structure of gp140.

To place the role of MPLA:phospholipid ratio in context, we, and to our knowledge others, have never used a liposomal MPLA:phospholipid ratio as high as 1:5.6 in an adjuvant formulation. Assuming that the average MW of MPL™ is 1605 [38] and of native MPLA is 1956 [14], then the MPLA:phospholipid ratio used with MPL™ for immunizing humans with R32NS1, a recombinant malaria antigen, was approximately1:73 [10]; and the ratio used for immunizing mice with a synthetic peptide antigen from gp41 of HIV-1 using native MPLA was approximately 1:244 [39]; and the MPLA:phospholipid ratio of the commercial AS01B adjuvant which contains MPL™ is approximately 1:41 [17].

Although we previously reported that a high MPLA:phospholipid ratio was associated with the possibility of a positive Limulus amebocyte lysate assay for detection of endotoxin [22], we have also previously demonstrated that so-called "limulus-positive" liposomes [40] do not necessarily cause rabbit or human pyrogenicity [10,41]. In the present instance, we determined under contract that ALF-type liposomes having an MPLA:phospholipid ratio of 1:8.8 were non-pyrogenic in a rabbit pyrogenicity assay conducted according to US FDA cGMP regulations (21 CFR Parts 210 and 211) (data not shown). This is further evidence that the region of the MPLA molecule responsible for pyrogenicity is more deeply buried in the hydrophobic region of the liposomal lipid bilayer than the region responsible for Limulus lysate activity.

Neutralizing antibodies were induced against CN54 gp140 in each of the two immunization experiments as detected by two clade C pseudoviruses but the neutralization titers were higher in the second of the two experiments. The ALF SUV with an MPLA:phospholipid ratio of 1:5.6 and ALFQ MLV with a ratio of 1:220 appeared to induce the strongest and most consistent NAbs. As previously reported [24,26,27,42], the NAbs that we observed had limited reactivities with other viruses. Neutralization was not observed either with a tier 2 clade C pseudovirus or with a tier 1 clade B pseudovirus (data not shown). The observations with ALFQ reinforce the possibility that induction of NAbs could have been due to unique structural interactions of liposomal ALFQ with gp140 rather than simple increased visibility of MPLA in the lipid bilayer.

The molecular relationships between different types of lipids at the surface of the liposomal bilayer can be unpredictable because of self-aggregation of individual lipid constituents, including the formation of lipid rafts, or as noted above because of the formation of non-lamellar polymorphisms of individual lipids such as MPLA [22]. In contrast to liposomes lacking QS21 different results were obtained with liposomes containing both MPLA and QS21 (i.e., ALFQ) in that high titers of antibodies occurred regardless of the ratio of MPLA:phospholipid. As discussed earlier, the structural changes responsible for the appearance of large particles in the presence of QS21 are not yet clear. Interestingly, the large sizes of many of the ALFQ particles that resulted in increased light scattering contrasted strongly with the reported uniform nano sizes of ISCOMS, ISCOMATRIX™, and Matrix-M™ and similar commercial vaccine adjuvant particles [43–45]. All of these latter types of particles contain *Quillaja* saponins (including QS21), Chol, and phospholipids, but they all lack MPLA. As noted above, we have previously described molecular interactions of QS21 with MPLA in liposomal membranes [22], and phospholipid type and cholesterol concentration of ISCOMS may play a role in size [46]. However, other than the presence of MPLA the reason for the large differences in particle sizes between ISCOM-type particles and ALFQ liposomal particles is not yet clear.

The present study demonstrates that the MPLA:phospholipid ratio and liposomal size are both important liposomal variables for induction of binding antibodies, IgG subtypes, and NAbs. Although this study involved female mice, it should be noted that the adjuvant effects might be different in males [47]. In addition, comparative adjuvant effects in mice do not predictably extrapolate to similar effects in humans [13,48]. Under the described conditions the immunological effects in this study occurred independently of the total dose of either the liposomal MPLA or the total dose of antigen, both of which were held constant. It is further noted that the ALFQ liposomes caused a distinctive induction of IFN-γ secretion that was independent of the MPLA:phospholipid ratio, and the ALFQ liposomes thus might be a potentially useful adjuvant for induction of Th1-type immunity. Interestingly, as shown in Fig. 3A, IgG2a was induced by SUV lacking QS21, resulting in an apparent relatively balanced Th1/Th2 response, but as shown in Fig. 4 the secretion of IFN-γ was low, possibly indicating a relatively weaker Th1 response. While this might seem a bit contradictory, it is known that secretion of IFN-γ is not an absolute requirement for induction of IgG2a antibodies and IgG2a antibodies can occur even in the complete absence of IFN-γ secretion [49,50]. Based on all of the above observations we are currently studying the effects

of liposomal size and MPLA:phospholipid ratios with ALF and ALFQ liposomes together with other antigens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.09.001.

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