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***In Vitro* Evaluation of TLR4 Agonist Activity: Formulation Effects**

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Abstract

Effective *in vitro* evaluation of vaccine adjuvants would allow higher throughput screening compared to *in vivo* studies. However, vaccine adjuvants comprise a wide range of structures and formulations ranging from soluble TLR agonists to complex lipid-based formulations. The effects of formulation parameters on *in vitro* bioactivity assays and the correlations with *in vivo* adjuvant activity is not well understood. In the present work, we employ the *Limulus* amebocyte lysate assay and a human macrophage cellular cytokine production assay to demonstrate the differences in *in vitro* bioactivity of four distinct formulations of the synthetic TLR4 agonist GLA: an aqueous nanosuspension (GLA-AF), an oil-in-water emulsion (GLA-SE), a liposome (GLA-LS), and an alum-adsorbed formulation (GLA-Alum). Furthermore, we demonstrate the importance of the localization of GLA on *in vitro* potency. By comparing to previous published reports on the *in vivo* bioactivity of these GLA-containing formulations, we conclude that the most potent activators of the *in vitro* systems may not be the most potent *in vivo* adjuvant formulations. Furthermore, we discuss the formulation considerations which should be taken into account when interpreting data from *in vitro* adjuvant activity assays.

Introduction

The evaluation of immune responses to vaccine adjuvants, including formulations of TLR ligands, can be approached by both *in vitro* and *in vivo* assays [1, 2]. The complexity of the immune response demands that some experimentation occur in relevant *in vivo* animal models, while keeping in mind the principle of the three R's of animal use (Refinement, Reduction and Replacement) [3]. The development of robust *in vitro* assays offers the benefit of replacement and can also be employed to evaluate certain aspects of adjuvants in a more time-efficient manner. However, the relative potency of particulate adjuvant formulations determined using *in vitro* assays of cytokine production are often not predictive of the relative potency *in vivo* when comparing across different formulation platforms. For instance, an aqueous nanosuspension of the synthetic TLR4 ligand glucopyranosyl lipid adjuvant (GLA) showed higher proinflammatory cytokine induction activity *in vitro* but lower antibody and cellular immune responses compared to oil-in-water emulsions of GLA [4].

TLR4 agonists such as GLA and other monophosphoryl lipid A analogues are promising vaccine adjuvant candidates to ensure a strong and specific immune response. The human

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papilloma virus vaccine Cervarix®, approved by the FDA in 2009, contains the TLR4 agonist MPL® formulated with aluminum oxyhydroxide. Triggering of TLR4 on the cell surface of antigen presenting cells results in the induction of innate and adaptive immune responses [5]. We have developed formulations of the synthetic TLR4 ligand GLA that enhance antibody and cellular immune responses when combined with recombinant or inactivated vaccine antigens in a formulation-dependent manner [2, 6, 7].

Particulate adjuvant formulations may differ in excipient composition as well as physicochemical properties. These differences in particulate formulations may affect active compound stability, facilitate controlled or targeted release, or enable formulation of insoluble molecules [8]. Several formulation platforms have been developed to incorporate GLA, including aqueous nanosuspensions, oil-in-water emulsions, liposomes, or aluminum salts [2, 6, 7]. Because each formulation contains different excipients that may have complex interactions with GLA, it is important to perform extensive analytical characterization in order to better understand such adjuvant-formulation interactions. In addition, the heterogeneity of dispersed-phased systems may also account for unforeseen interactions with GLA. For example, oil-in-water emulsions are not just uniform emulsified oil droplets; they also contain other structures, such as liposomes, micelles, and monomers [9]. Several analytical techniques are useful for analysis of GLA-containing formulations. Examples include dynamic light scattering and HPLC with charged aerosol detection (CAD), which provide essential data regarding particle size and chemical stability or adjuvant concentration.

In the present work, we sought to evaluate the effects of different formulations on TLR4 ligand (i.e. GLA) *in vitro* activity. We hypothesized that differences in *in vitro* adjuvant activity may be due to formulation effects on the *in vitro* availability of GLA rather than on actual differences in adjuvant potency. Five distinct formulations of GLA were employed: aqueous nanosuspension (GLA-AF), stable emulsion (GLA-SE), liposomal (GLA-LS), alum-adsorbed (GLA-Alum), and a mixture of GLA-AF and stable emulsion (GLA-AF + SE). To evaluate *in vitro* biological activity of the GLA formulations, we have employed two distinct assays responsive to TLR4 ligands: 1) the Limulus Amebocyte Lysate (LAL) assay which is based on the reaction between lipopolysaccharide (the parent molecule from which monophosphoryl lipid A molecules are derived) and a clottable protein in the circulating amebocytes of *Limulus polyphemus* [10, 11] and 2) cytokine production in a human acute monocytic leukemia-derived cell line (Mono Mac 6) [12].

Materials and Methods

Adjuvant Formulation Materials and Manufacture

Synthetic monophosphoryl lipid A (glucopyranosyl lipid adjuvant or GLA) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC). were purchased from Corden Pharma (Liestal, Switzerland), NOF American Corp. (Irvine, CA), or Avanti Polar Lipids. Squalene was purchased from Sigma-Aldrich (St. Louis, MO). Poloxamer 188 and glycerol were purchased from Spectrum Chemical (Gardena, CA). Cholesterol, monobasic and dibasic ammonium phosphate were purchased from J.T. Baker (San Francisco, CA). Phosphate buffered saline 1x (PBS) at pH 7.2 was purchased from Invitrogen (Grand Island, NY). Alhydrogel® 85 (aluminum oxyhydroxide gel) was purchased from E.M. Sergeant Pulp & Chemical Co. (Kalamazoo, MI).

GLA-AF was manufactured by first combining DPPC and GLA (a 2:1 DPPC:GLA molar ratio) in chloroform, which was then evaporated using a Buchi Rotovapor R-114 (Flawil,

Switzerland). The dried DPPC and GLA were rehydrated in ultrapure water, then sonicated in a VWR 75D (West Chester, PA) or Crest Powersonic CP230D (Trenton, NJ) sonicating water bath at ~60°C for ~1–2 hours or until the formulation was translucent with no visible particles.

GLA-SE was manufactured by mixing a buffered aqueous phase and an oil phase with a Silverson Heavy Duty Laboratory Mixer Emulsifier (3/4 in. tubular square hole high shear screen attachment; East Longmeadow, MA) at ~7,000 – 10,000 rpm for ~10 minutes, then microfluidizing the mixture using the Microfluidics M110P (Newton, MA) for ~12 passes at 30,000 psi. For a 200 mL total batch volume, the buffered aqueous phase was first prepared by combining 0.18 g poloxamer 188 and 4.5 g glycerol in 180 ml of 25 mM ammonium phosphate buffer. The oil phase was prepared by dispersing 3.8 g DMPC and 0.05 g GLA into 20 ml of squalene. The oil phase was sonicated in a VWR 75D or Crest Powersonic CP230D sonicating water bath at ~70°C for 2–3 hours or until the PC and GLA were evenly dispersed. In batches of SE, GLA was omitted, as indicated below.

GLA-LS was manufactured by first combining 1.8 g DPPC, 0.5 g cholesterol, 0.2 g DPPG, and 25 mg GLA in 5 ml of 2:1 chloroform:methanol, which was then evaporated using a Buchi Rotovapor R-114 (Flawil, Switzerland). The dried components were rehydrated in 100 ml PBS (pH 7.2), then sonicated in a VWR 75D or Crest Powersonic CP230D sonicating water bath ~60°C until all the solids were suspended in the buffer. The mixture was then transferred to the Microfluidics M110P (Newton, MA) for high-pressure homogenization for 12 passes at 10,000 psi. GLA-Alum was manufactured by mixing GLA-AF with Alhydrogel® 85 for a final aluminum content of 2 mg/ml. GLA-AF + SE was manufactured by mixing GLA-AF with SE at a 1:1 volume ratio, immediately before performing the *in vitro* assays. All formulations were stored at 5 °C.

Dynamic Light Scattering Particle Size Analysis

Particle size for all formulations was determined using the Malvern Instruments (Worcestershire, UK) Zetasizer Nano-S via dynamic light scattering (DLS). For GLA-SE, GLA-LS, and SE, 5 µl of formulation were combined with 500 µl ultrapure water in a 1.5-ml polystyrene disposable cuvette. For GLA-AF, 50 µl of formulation were combined with 450 µl ultrapure water in a 1.5-ml polystyrene disposable cuvette. For each formulation tested, three separate cuvettes were prepared. All DLS measurements were then made three times with each of the three separate cuvettes. In order to examine the size of the formulations after diluting into cell culture media, all formulations were mixed with the media before particle size was determined. For GLA-AF, GLA-LS, GLA-SE, and SE, 25 µl of formulation were combined with 475 µl cell culture media in a 1.5-ml polystyrene disposable cuvette and then measured for particle size by DLS. In this case, for each formulation tested one cuvette was prepared, with which DLS measurements were made three times.

Nanoparticle Tracking Particle Size Analysis

Particle size distributions and particle concentration were evaluated for each class of formulations using a NanoSight LM10 nanosizer with a 40 mW 410 nm laser (Wiltshire, UK) and a Hamamatsu Orca Flash 2.8 CMOS camera (Hamamatsu, JP). Before and after each measurement the sample cell was rinsed with three cell volumes ultrapure water filtered through a 20-nm Anotop 25 Plus filter (Whatman, UK). Samples were diluted 1:10⁴ (GLA-AF) or 1:10⁶ (GLA-SE, GLA-LS) in 20-nm filtered ultrapure water in two or three steps. Each formulation was diluted four times, independently, to account for dilution error. Ninety seconds of video were recorded for each sample with optimized shutter and gain

settings. The camera histogram gating was adjusted to maximize sensitivity. Data analysis was performed in NanoSight NTA 2.3 (Wiltshire, UK) in standard mode.

HPLC-CAD Analysis

To prepare GLA-SE and GLA-LS, 50 μ l of formulation were combined with 950 μ l mobile phase B (1:1 [v/v] methanol:chloroform with 20 mM ammonium acetate and 1% acetic acid) in 1.5-ml glass vials. To prepare GLA-AF samples, 50 μ l of formulation were combined with 950 μ l mobile phase A (75:15:10 [v:v:v] methanol:chloroform:water with 20 mM ammonium acetate and 1% acetic acid). For each formulation, three separate vials were prepared. All samples were injected on a Waters Co. (Milford, MA) Atlantis T3 column attached to an Agilent Model 1100 HPLC (Santa Clara, CA). A gradient consisting of mobile phases A and B was used over 25 minutes. Detection was performed by an ESA Biosciences (Chelmsford, MA) Corona Charged Aerosol Detector (CAD). Quantitation was performed using GLA standards at 100, 80, 60, 40, 20, 16, 12, 8, 4, 2, 1 μ g/ml in mobile phase B.

LAL Assay

The Limulus Amebocyte Lysate (LAL) assay was purchased from Lonza (QCL-1000, Walkersville, MD). GLA-containing formulations were evaluated using the LAL assay according to the manufacturer instructions. Briefly, samples and standards were diluted in LAL-reagent water and placed in a 96-well microplate. Each sample was assayed in duplicate or triplicate. A spike of 0.4 endotoxin units (EU)/ml (*E. coli* endotoxin strain 0111:B4) was added to the samples to check for inhibition or enhancement of the assay. LAL reagent and chromogenic substrate were added to the plate and the reaction was allowed to proceed at 37 °C. Plates were monitored for 405-nm wavelength absorbance. Statistical comparisons, employing ANOVA with Tukey's multiple group comparison test or student's t-test on log-transformed values, were carried out using GraphPad Prism 5 (San Diego, CA).

Mono Mac 6 Cytokine Assay

Mono Mac 6 cells were obtained from DSMZ (ACC 124, Braunschweig, Germany). Human CCL2 (MCP-1) ELISA kit was purchased from eBioscience (product 88-7399, San Diego, CA) and Human CCL4/MIP-1 β ELISA kit was purchased from R&D Systems (product DY271, Minneapolis, MN). Mono Mac 6 cells were plated at 1×10^5 cells per well in a 96-well plate. GLA formulations were added at concentrations from 25 to 5000 nM (typical human dose of GLA in various clinical trials has ranged from 2 to 10 μ M). After incubation for 24 hours at 37 °C, the supernatants were assayed for secretion of MIP1- β and MCP-1 cytokines by ELISA. MIP1- β or MCP-1 capture antibodies were bound to Costar EIA/RIA 96 well plates (Corning, NY). After an overnight incubation, blocking solution was added and incubated for 1 hour at room temperature. Cell supernatants at a 1:20 dilution for each cytokine assay were added to the plates, along with MCP-1 and MIP-1 β protein standards. After a 2 hour incubation, detection antibodies were added (1 hour incubation), followed by streptavidin/HRP (30 min incubation). Three washes with PBS-0.1% Tween 20 were performed between each incubation step. TMB substrate (eBioscience) was added for color development and the reaction was stopped with 1N sulfuric acid. Absorbance was measured at 450 nm. The viability of the Mono Mac 6 cells after the 24 hour incubation with GLA formulations was measured by the Guava ViaCount Assay, at the highest concentration of formulation tested (5000 nM).

Results

Formulation Composition and Characterization

The different formulations manufactured for this study vary significantly in physicochemical attributes. GLA-AF, containing a minimum of excipients, is an aqueous nanosuspension of lipid micelles and related aggregate structures. GLA-SE contains oil droplets emulsified with phospholipid and block copolymer. GLA-LS consists of spherical bilayer vesicles formed by the self-assembly of phospholipids. GLA-Alum represents GLA-AF particles adsorbed to aluminum oxyhydroxide gel, which itself consists of crystalline aggregates in the low micron size range [13]. Formulation composition and physicochemical characteristics are shown in Table 1. Average particle size based on ensemble fluctuations in scattering intensity (Z-ave), size polydispersity (PdI), and GLA concentration were assessed by dynamic light scattering and HPLC-CAD, respectively, in four separate batches of GLA-AF, and one batch each of GLA-SE and GLA-LS, as shown in Table 1. Particle size and particle concentration of selected formulations were also determined by a relatively new technique called nanoparticle tracking analysis, which is based on the Brownian motion of individual particles tracked by light microscopy (Table 1) [14]. The GLA-Alum formulation was not characterized for particle size (due to the large and heterogeneous size of the particles in Alhydrogel®) nor was this formulation conducive to direct quantitation by HPLC-CAD (due to the high affinity adsorption of GLA to Alhydrogel®), although previous analysis of supernatant of centrifuged GLA-Alum indicates complete adsorption of GLA [13]. Both sizing methods produced similar results, with the average particle size values of GLA-AF, GLA-SE, and GLA-LS in the ~75–90 nm range, although GLA-AF and GLA-LS have greater size polydispersity than GLA-SE (Table 1 and Supplementary Information). GLA-SE and GLA-LS particle concentrations were 3.5×10^{14} and 1.8×10^{14} particles/ml respectively while GLA-AF formulations averaged $\sim 1.8 \times 10^{12}$ particles/ml. The number of particles correlates well with the expected formulation structure: for instance, GLA-LS contains about half the number of particles as GLA-SE even though both contain about the same concentration of phospholipid; this is due the emulsion consisting of phospholipid monolayers (surrounding oily cores) whereas liposomes consist of phospholipid bilayer vesicles. Some GLA-AF batches were manufactured at different GLA concentrations, but size values were reproducible and all batches were diluted to the same stock concentration prior to employing them in the *in vitro* assays.

Besides composition, another key difference between the various formulations is particle size. Although the intensity-based average size values are similar between GLA-AF, GLA-SE, and GLA-LS, the actual size composition requires a more nuanced discussion. The intensity-based particle size distribution is biased towards larger particles due to their enhanced ability to scatter light. Volume-based size distributions, obtained by a mathematical conversion, are less influenced by larger particles. The intensity-based and volume-based size distributions of GLA-AF, GLA-SE, and GLA-LS are shown in Figure 1. The volume-based size distribution of GLA-AF indicates a high ratio of smaller particles compared to GLA-LS or GLA-SE. Variation in processing methods may also account for some of the size differences. While GLA-SE and GLA-LS were subject to high pressure homogenization, GLA-AF was processed via sonication. Figure 5 also indicates that a small population of larger particles ($>1 \mu\text{m}$) were present in GLA-LS.

LAL Assay

The LAL method is widely used as a quantitative test for gram-negative endotoxin (i.e. lipopolysaccharide [LPS]). Compared to LPS, vaccine adjuvants based on the monophosphoryl lipid A structure show dramatically reduced *in vivo* toxicity [15]. However, due to the similarity in structure between LPS and monophosphoryl lipid A molecules, the

latter are also activators of the LAL assay. The LAL response to endotoxin is gel clotting, with LAL clotting enzyme releasing the chromogenic p-nitroaniline (pNA) from a synthetic substrate, where the cleaved pNA is measured photometrically [16]. We first examined the ability of the LAL assay to reproducibly detect GLA when formulated as an aqueous nanosuspension (GLA-AF). Serial dilutions of four different batches of GLA-AF were made in LAL-reagent water and measured in the dilution range of 4×10^{-6} to 3.2×10^{-7} by the LAL assay (Figure 2A). With each batch assayed in duplicate or triplicate, the combined average ($\pm$ s.d.) result of GLA-AF was 1.57×10^7 (± 0.43) EU per mg of GLA.

We next examined whether the ability of the LAL assay to detect TLR4 agonist content was affected by different formulations of GLA. Therefore, we compared the above LAL assay results regarding GLA-AF to GLA that had been formulated in oil-in-water emulsion (GLA-SE), liposome (GLA-LS), or adsorbed to Alhydrogel® (GLA-Alum); see Figure 2B. In each of these formulations, the apparent GLA content registered in the LAL assay is significantly ($p < 0.05$) less than in the aqueous nanosuspension (GLA-AF). Furthermore, the GLA-LS formulation indicated (apparently) significantly ($p < 0.05$) less GLA than the GLA-Alum formulation. Since HPLC-CAD analysis indicates that each formulation has the same concentration of GLA, it is apparent that the formulation affects the LAL assay. To test whether formulation excipients were interfering with the LAL assay, we prepared a novel formulation consisting of GLA-AF admixed with oil-in-water emulsion (SE) immediately before the LAL assay. This formulation (GLA-AF + SE) was made to contain the same amount of GLA as a corresponding GLA-SE. The difference is the localization of the GLA. In GLA-SE, the GLA is suspected to integrate at the interface of the oil-in-water emulsion [4], whereas in freshly prepared GLA-AF + SE the GLA is expected to be present in the aqueous phase as a nanosuspension. The LAL assay results comparing GLA-AF and GLA-AF + SE are included in Figure 2B. There is no difference in the detected GLA content in GLA-AF versus GLA-AF + SE. As expected, the negative control SE shows negligible contribution. These results indicate that the excipients present in the emulsion are not interfering or contributing to the assay per se, but rather affect the availability of the GLA in the LAL assay such that when formulated as GLA-SE, lower amounts of GLA are detected than when formulated as GLA-AF. Note that in all assays run, no inhibition or enhancement of the LAL test was observed using spike recovery criteria of 50–200% as determined by the test manufacturer.

Mono Mac 6 Cytokine Assay

To determine the effects of TLR4 ligand formulation on another type of *in vitro* bioactivity assay besides the LAL assay, we employed the human acute monocytic leukemia derived cell line, Mono Mac 6. The Mono Mac 6 cell line, as opposed to other monoblast lines such as U937 or THP-1, exhibits characteristics of mature monocytes [12]. Moreover, the Mono Mac 6 line has been used in evaluation of adjuvant activity in the literature, notably by Zaitseva et al. as an *in vitro* model of adjuvant safety. Cells were stimulated with GLA-AF, GLA-SE, GLA-LS, GLA-Alum, or GLA-AF + SE and production of MCP-1 and MIP-1 β cytokines was monitored by ELISA of the supernatants of incubated samples. MCP-1 (monocyte chemotactic protein-1 or CCL2) attracts monocytes, NK and T cells, and dendritic cells to infection sites, and is associated with both Th1- and Th2-type immune responses whereas MIP-1 β (macrophage inflammatory protein-1 β or CCL4) is another chemokine attracting similar cell populations as MCP-1 but is more associated with Th1-type immune responses [19, 20]. Due to its linearity and reproducibility, Mono Mac 6 with MIP-1 β readout is being developed in our laboratory as a biological release assay of GLA-AF.

A GLA concentration range from 25 to 5000 nM for each formulation (except the control SE) was prepared by serial dilution. The most potent responses at all dilutions as measured by MCP-1 and MIP1- β cytokine secretion are evident with GLA-AF and were consistent for all four batches of GLA-AF (Figure 3; also see Supplementary Figure 3A for comparison of GLA-AF to the diphosphorylated Lipid A 506). In contrast, GLA-LS, GLA-Alum, and SE control induced negligible cytokine induction. The GLA-AF + SE formulation shows a trend of inducing somewhat higher levels of MCP-1 and MIP-1 β compared to the other formulations (excepting GLA-AF), although the variation in the data is large for MIP-1 β which may hint at complex interactions between the freshly mixed SE and the GLA-AF. However, at high GLA concentrations, the cytokine response elicited by GLA-AF + SE is dampened. The mechanism for the reduced cytokine response at high GLA concentrations in GLA-AF + SE is unclear; it does not appear that this is due to interference from SE excipients since the SE control does not show a dose response effect. Likewise, the viability of the cells was only slightly affected or not affected at all by the presence of any of the formulations, with >74% viability maintained for each formulation tested (Supplementary Figure 3B).

We also investigated whether formulation particle size changed after dilution into the Mono Mac 6 cell culture media and found that the original sizes of the GLA-SE and GLA-LS formulation were maintained (Supplementary Figure 3C); however the interpretation of the GLA-AF size profile is not possible due to the interference of a large population of particles from the constituents of the cell culture media itself that may overpower the scattering from the GLA-AF particles, which are some two orders of magnitude fewer in number compared to the other formulations (Table 1). Finally, to illustrate that the cytokine production patterns evident in the Mono Mac 6 cell line are not exclusive to the cell line, we examined a subset of formulations with regards to their effects on MIP-1 β and MCP-1 production on human whole blood (Supplementary Figure 3D); overall patterns were similar with the exceptions that the GLA dose dependence was more nuanced between GLA-SE and GLA-AF with regards to MCP-1, and overall GLA-SE elicited higher cytokine responses in the whole blood assay compared to GLA-SE in the Mono Mac 6 cell line.

Discussion

Structural differences between the different formulations of GLA appear to have critical effects on the ability of GLA to activate the *in vitro* assays shown here. GLA-AF contains very little excipient compared to the other formulations, which means that each particle in GLA-AF contains a high amount of GLA relative to the phospholipid excipient, DPPC. GLA-SE and GLA-LS, on the other hand, contain ~100 fold more phospholipid and additional excipients compared to GLA-AF. In effect, this means a larger number of particles and lower amount of GLA per particle in the GLA-SE and GLA-LS formulations. Indeed, nanoparticle tracking analysis indicates that GLA-SE and GLA-LS have at least 100-fold more particles than GLA-AF (Table 1), although it should be noted that the smaller particles in GLA-AF (see discussion below) may not be detectable by this method, which is less accurate for weakly scattering particles smaller than ~30 nm [14]. In the case of GLA-Alum, which consists of GLA-AF adsorbed to aluminum oxyhydroxide, the GLA-AF may not be readily available for *in vitro* reactions due to its high affinity for the aluminum oxyhydroxide particles. Likewise, GLA-LS and GLA-SE could have higher-affinity matrices for GLA compared to that of GLA-AF. This conclusion is in line with previous work on the masking effect of liposomes incorporating LPS on the LAL assay, which concluded that the liposome-sequestered LPS was unavailable for LAL detection until detergent-based solubilization and/or other processing steps were accomplished [21, 22]. In any case, the smaller number of particles in GLA-AF means that significantly more molecules of GLA are present per particle compared to the other formulations (Table 1).

Thus, it is possible that the smaller sizes in GLA-AF and/or the high ratio of multimeric GLA per particle are more active or more available in the *in vitro* assays.

The ability of the LAL assay to reproducibly detect GLA content in different batches of the same formulation indicates its potential to be used as a quantitative tool to detect low concentrations of TLR4 agonist. This is especially important considering the sensitivity limitations of current HPLC-based assays. For example, the estimated limit of detection for HPLC-CAD analysis of GLA is 0.5 $\mu\text{g/ml}$ [4], whereas the diluted concentration of GLA in the LAL assay results shown in Figure 1 was on the order of ~ 25 pg/ml, a 20,000-fold increase in sensitivity.

Taken together, these data confirm that formulation effects of TLR4 ligands are essential factors in interpreting *in vitro* bioactivity. Interestingly, a recent report that the production of pro-inflammatory cytokines is greatly increased when Mono Mac 6 cells are stimulated with aqueous-based formulations of TLR agonists (FSL-1, Pam3CSK4, flagellin and R848) compared to clinical adjuvants (Alum, MF59, MPL and ISCOMATRIX) did not consider the effects that formulation could have on either the *in vitro* or *in vivo* bioactivities, even though the studied adjuvants/TLR agonists vary significantly, from soluble molecules like R848 to complex lipid matrices like ISCOMATRIX [23]. In contrast, Seydel, Brandenburg and colleagues have conducted extensive investigations showing the importance of molecular structure and formulation aspects, including geometry, aggregation, and formulation preparation methods, on *in vitro* bioactivity of LPS-related structures and lipopeptides [24, 25], including pointing out that correlations between LAL and cellular cytokine assays may or may not correlate depending on the structure of the LPS derivative [26]. Moreover, nanoparticles of different structures may interfere or even enhance *in vitro* LAL assay results [27, 28]. Thus, it is clear from the present findings and a review of the literature that formulation factors that may affect *in vitro* and *in vivo* bioavailability include size, multimeric presentation, molecule localization, and/or molecule-particle affinity.

Most importantly, *in vitro* comparisons of the relative potency of different formulations of TLR4 ligands may not be predictive of relative potency *in vivo*, especially if comparing across different formulation platforms. For example, whereas GLA-AF is clearly the most potent activator in the LAL and cytokine assays *in vitro* compared to GLA-SE, this may not be the case for *in vivo* immunogenicity [4, 6, 29, 30]. Furthermore, whereas in the present work the GLA-Alum formulation appeared more potent in the LAL assay than the GLA-SE formulation, the opposite was true for *in vivo* immunogenicity evaluation with a malaria antigen [7]. Table 2 compiles several published results comparing the bioactivity of formulations of GLA combined with different vaccine antigens and routes of administration. Thus, formulations which induce low bioactivity in *in vitro* assays such as those shown here may be highly efficacious *in vivo* due to enhanced stability, controlled release of TLR4 agonist, or other formulation-dependent characteristics not represented accurately *in vitro*. Therefore, *in vitro* comparative analysis may be more useful when comparing different immunostimulators formulated in a common platform.

Whereas *in vitro* analysis of the immune system has allowed us to quantitatively measure specific aspects of immune responses such as cytokine release or adjuvant uptake, these assays are usually limited to a single cell type (e.g. Mono Mac 6) or subset of cells (e.g. PBMCs). Thus, the complexities of human immunophysiology systems, including three dimensional anatomy, extracellular milieu, and nonequilibrium flow dynamics are poorly modeled by *in vitro* systems [31]. For this reason, appropriate animal models are employed to help bridge the gap between *in vitro* experiments and human disease. These considerations should be taken into account when designing and interpreting *in vitro* adjuvant activity assays. Furthermore, more work is needed to understand the effects of

formulation factors on *in vitro* assays so that they can be more effectively employed as replacements for *in vivo* studies by incorporating aspects such as three-dimensional design with improved mimicry of the *in vivo* environment [32]. For example, in a human skin explant model with intradermal injection of GLA-containing formulations, GLA-SE enhanced dermal dendritic cell cytokine activation and T cell proliferation compared to GLA-AF (or LPS) [33]. Moreover, in elderly human PBMCs challenged *ex vivo* with live influenza virus, enhanced Granzyme B activity (indicative of cytotoxic T lymphocyte activity) was demonstrated after exposure to GLA-SE, but not GLA-AF, when administered with split virus vaccine [34]. Thus, more complex *in vitro* analyses such as those cited may be more informative and/or predictive of *in vivo* bioactivity when comparing different formulations of a TLR ligand such as GLA. In conclusion, *in vitro* analysis of adjuvant bioactivity can be substantially informative, but should be interpreted with caution when comparing across different formulation platforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

TLR4 ligands are promising vaccine adjuvant candidates

In vitro assays (e.g. macrophage cytokine production) evaluate adjuvant activity

Different formulations of the same TLR4 ligand modulate its *in vitro* bioactivity

Figure 1A

Figure 1B

Figure 1. (a) Intensity-based size distributions of GLA-containing formulations. (b) Volume-based size distributions of GLA-containing formulations.

Figure 2A

Figure 2B

Figure 2.

(a) Detection of GLA in four separate batches of aqueous nanosuspension by the LAL assay. Error bars represent standard deviation from 2–5 replicates for each formulation in the LAL assay. (b) Detection of GLA as aqueous nanosuspension (GLA-AF), stable emulsion (GLA-SE), liposome (GLA-LS) adsorbed to alum (GLA-Alum), aqueous nanosuspension post-mixed to emulsion (GLA-AF + SE), or emulsion without GLA (SE) by the LAL assay. The GLA-AF data is compiled from Figure 2a, representing four separate batches of GLA-AF, whereas the other formulations contain 2 or 3 batches of each formulation (each of which was assayed at least twice) with error bars representing standard deviation. SE does not contain GLA but is provided as a control to the GLA-AF + SE group. * $p < 0.05$ vs. GLA-AF, † $p < 0.05$ vs. GLA-SE, ^ $p < 0.05$ vs. GLA-LS, # $p < 0.05$ vs. GLA-Alum, § $p < 0.05$ vs. GLA-AF + SE.

Figure 3A

Figure 3B

Figure 3.

(a) MCP-1 cytokine responses from Mono Mac 6 cells upon stimulation with GLA-containing formulations. GLA molar concentrations are represented in the legend (with the exception that SE does not contain GLA but is provided as a control to the GLA-AF + SE group). Error bars represent standard deviation from at least two distinct batches of each formulation. (b) MIP-1 β cytokine responses by Mono Mac 6 cells upon stimulation with GLA-containing formulations. GLA molar concentrations are represented in the legend (with the exception that SE does not contain GLA but is provided as a control to the GLA-AF + SE group). Error bars represent standard deviation from at least two distinct batches of each formulation.

Composition, particle size and GLA concentration of tested formulations.

Table 1

Formulation	Composition	Dynamic Light Scattering		Nanoparticle Tracking Analysis			Measured GLA Concentration (µg/ml)	Dilution Factor	Calculated GLA concentration (µg/ml)	Estimated per-particle GLA concentration (molecules/particle ± %error)
		Particle size (Z-ave, nm)	PdI	Particle Size (Mean Diameter, nm)	Size Distribution Standard Deviation (nm)	Particle Concentration (particles/ml)				
GLA-AF(1)	GLA, DPPC	90 ± 2	0.232 ± 0.007	89 ± 1	40 ± 4	2.19 ± 0.09 × 10 ¹²	201	None	201	34000 ± 6%
GLA-AF(2)*		76 ± 1	0.247 ± 0.006	NA	NA	NA	659*	1:2.5	264	NA
GLA-AF(3)*		85 ± 1	0.284 ± 0.008	90 ± 10	40 ± 20	1.31 ± 0.04 × 10 ¹²	992*	1:4	248	65000 ± 3%
GLA-AF(4)*		93 ± 2	0.285 ± 0.003	NA	NA	NA	732	1:2.9	252	NA
GLA-SE(1)	GLA, squalene, DMPC, poloxamer 188, glycerol, ammonium phosphate buffer	76 ± 1	0.057 ± 0.019	84 ± 3	24 ± 3	3.5 ± 0.2 × 10 ¹⁴	251	None	251	240 ± 6%
GLA-SE(2)		92 ± 2	0.048 ± 0.014	NA	NA	NA	264	None	264	NA
GLA-LS(1)	GLA, DPPC, DPPG, Cholesterol, PBS	91 ± 2	0.245 ± 0.006	85 ± 5	34 ± 9	1.8 ± 0.2 × 10 ¹⁴	216	None	216	470 ± 11%
GLA-LS(2)		79 ± 1	0.140 ± 0.013	NA	NA	NA	230	None	230	NA
GLA-Alum(1)	GLA, DPPC, Alhydrogel®	NA	NA	NA	NA	NA	NA	None	250	NA
GLA-Alum(2)										
GLA-Alum(3)										
SE(1)	squalene, DMPC, poloxamer 188, glycerol, ammonium phosphate buffer	82 ± 1	0.044 ± 0.013	NA	NA	NA	NA	None	NA	NA
SE(2)		83 ± 1	0.044 ± 0.010	NA	NA	NA	NA	None	NA	NA

* Some GLA-AF formulations were made at higher stock concentrations, but were diluted to be equivalent to the other formulations prior to *in vitro* assays.

Table 2
Summary of published preclinical *in vivo* comparisons of GLA-containing formulations

Formulations tested	Vaccine antigen	Animal model	Immunization route	GLA dose (μ g)	Summary of comparative findings	Reference
GLA-AF, GLA-SE	LmSTII (Leishmaniasis)	Mouse (BALB/c)	Subcutaneous	20	GLA-SE elicited higher IgG2a/IgG1 antibody ratio compared to GLA-AF, and there was a trend for higher total IgG and IFN- γ production in GLA-SE groups but results were not always statistically significant	4
GLA-AF, GLA-SE	PfCelTOS (Malaria)	Mouse (BALB/c)	Subcutaneous	5	GLA-SE and GLA-AF elicited similar IgG1 and IgG2a antibody titers and long-lived antibody-secreting plasma cells, but only GLA-SE enhanced IFN- γ and IL-17 levels compared to antigen alone	6
GLA-Alum, GLA-SE	GMZ2 (Malaria)	Mouse (CB6F1)	Subcutaneous	20	GLA-Alum and GLA-SE induced similar IgG, IgG1, IgG2b, and IFA titers, and long-lived antibody-secreting plasma cells, with a trend towards higher IgG2a antibody titers and higher IFN- γ elicited by GLA-SE	7
GLA-AF, GLA-SE	Fluzone® (Influenza)	Mouse (BALB/c)	Intramuscular	20	GLA-SE and GLA-AF induced similar total IgG and IgG1 antibody titers, but GLA-SE elicited enhanced IgG2a antibody titers, HI titers, and IFN- γ and IL-2 production compared to GLA-AF	29
GLA-AF, GLA-Alum, GLA-LS, GLA-SE	ID93 (Tuberculosis)	Mouse (C57BL/6)	Intramuscular	5	All GLA-containing formulations enhanced IgG2a antibody titers to similar levels; GLA-SE induced the highest Th1-type cytokines (IFN- γ , TNF- α , IL-2), although GLA-LS and GLA-Alum (but not GLA-AF) also induced significant levels of Th1-type cytokines; GLA-SE, GLA-LS, and GLA-Alum (but not GLA-AF) provided protection from TB challenge	30
GLA-AF, GLA-SE	gp140 (HIV)	Mouse (C57BL/6)	Intranasal or Intramuscular	10	GLA-AF (intranasal), GLA-AF (intramuscular), and GLA-SE (intramuscular) enhanced total IgG to similar levels and IgG2c/IgG1 ratios were also similar; GLA-AF (intramuscular) enhanced long-lived antibody-secreting plasma cells more than GLA-SE (intramuscular)	35