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Glucopyranosyl lipid adjuvant enhances immune response to Ebola virus-like particle vaccine in mice

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ABSTRACT

The identification of adjuvants that promote lasting antigen-specific immunity and augment vaccine efficacy are integral to the development of new protein-based vaccines. The Ebola virus-like particle (VLP) vaccine expressing Ebola virus glycoprotein (GP) and matrix protein (VP40) was used in this study to evaluate the ability of TLR4 agonist glucopyranosyl lipid adjuvant (GLA) formulated in a stable emulsion (SE) to enhance immunogenicity and promote durable protection against mouse-adapted Ebola virus (ma-EBOV). Antibody responses and Ebola-specific T cell responses were evaluated post vaccination. Survival analysis after lethal ma-EBOV challenge was performed 4 weeks and 22 weeks following final vaccination. GLA-SE enhanced EBOV-specific immunity and resulted in long-term protection against challenge with ma-EBOV infection in a mouse model. Specifically, GLA-SE elicited Th1-skewed antibodies and promoted the generation of EBOV GP-specific polyfunctional T cells. These results provide further support for the utility of TLR4 activating GLA-SE-adjuvanted vaccines.

1. Introduction

Protein-based vaccines promote protection by targeting relevant viral protein antigens. They do not require propagation of infectious material and do not rely on attenuation of pathogens that may revert to virulence, making them a safer platform option. Virus-like particles (VLPs) are non-infectious particles that resemble virus; they contain essential antigenic proteins but lack viral genomic material. The use of VLP as a delivery system for protein antigens permits the preservation of antigen structure, which may be essential for antigen processing and presentation. Self-assembled VLP vaccines are currently in use for the prevention of Hepatitis B and human papillomavirus (HPV) [1–3].

EBOV VLPs are multiprotein structures that mirror the morphology of authentic Ebola virus without the presence of the viral genome [4]. Formation of EBOV VLPs can be induced by transfection of human or insect cell lines with the genes encoding glycoprotein (GP) and structural protein, VP40 [5–8]. EBOV VLP has been reported to be protective in mouse, guinea pig, and non-human primate models of EBOV infection [6,8–11]. When used as monotherapy, protein-based vaccines, such as VLPs, may be less immunogenic and responses are often less durable compared to live-attenuated formulations [12]. To overcome these challenges, adjuvants are frequently incorporated into protein-based VLP vaccine platforms.

Adjuvants are immunological or pharmacological agents that have the ability to enhance vaccine-induced immune responses. Currently, several types of adjuvants have been employed in vaccines approved by the US Food and Drug Administration (FDA). These adjuvants consist of different components, such as the following: aluminum salts; emulsions, such as MF59 and AS03; Toll-like receptor (TLR) agonists (CpG ODN or monophosphoryl lipid A (MPL) adsorbed on aluminum salts as in AS04); or a combi-

Abbreviations: GLA, glucopyranosyl lipid adjuvant; SE, stable emulsion; VLP, virus-like particle; EBOV, Ebola virus; Th, T helper; CD, cluster of differentiation; ELISA, enzyme-linked immunosorbent assayBME-beta mercaptoethanol; ELISPOT, enzyme-linked immunosorbent spot; DMSO, dimethyl sulfoxide; FACs, fluoresce active cell sorting; ICS, intra-cellular staining; FBS, fetal bovine serum; GP, glycoprotein; VP, viral protein; IACUC, Institutional Animal Care and Use Committee; IM, intramuscular; IP, intraperitoneal; Ma, EBOV-mouse-adapted Ebola virus; MPL, Monophosphoryl Lipid A; APC, antigen presenting cells; pfu, plaque forming unit; TLR, Toll-like receptor; USAMRIID, United States Army Medical Institute of Infectious Disease; HPV, human papillomavirus; AS03, Adjuvant system 3; CpG-ODN-CpG, oligonucleotides; CO2, carbon dioxide; IFNγ, interferon gamma; HRP, horseradish peroxidase; AF, aqueous formulations; FDA, Food and Drug Administration; PMA, 12-myristate 13-acetate.

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nation of immunopotentiators (QS-21 and MPL in AS01) [13]. However, adjuvanticity may be impacted by stability, formulation, or the vaccine platform to which the adjuvant is partnered. Historically, adjuvants formulated with aluminum have resulted in loss of vaccine immunogenicity due to freezing or lyophilization. Additionally, aluminum-containing adjuvants are effective at stimulating T helper (Th) type 2-biased immune responses but may not induce the required immunological effects to control certain intracellular pathogens, specifically Ebola virus [14–16]. While MPL incorporation has been shown to be effective in vaccines for HPV, previous mouse studies have demonstrated that vaccination with MPL-adjuvanted EBOV VLP offered only partial protection against ma-EBOV [11]. To this end, additional research to identify optimal immunostimulatory adjuvants with proven clinical safety that promote the induction of effective EBOV VLP vaccine-induced immune responses is critical for the advanced development of the vaccine.

TLR agonists exert adjuvant function through directly stimulating receptors found on innate immune cells to potentiate inflammatory responses pathways [12,14,17,18]. Signal transduction pathways activated by TLR agonists augment antigen presenting cell (APC) function to target the appropriate adaptive immune response required for prolonged vaccine efficacy [19–21]. Notably, glucopyranosyl lipid adjuvant (GLA) is a synthetic TLR4 agonist that is formulated in a stable oil-in-water emulsion (SE) containing squalene. It has been reported that GLA-SE promotes the induction of Th1 immunity, which is protective against viral infections [22].

In this study, the impact of combining GLA-SE with EBOV VLP was evaluated. The ability of GLA to enhance VLP-induced immunity and promote durable protection against EBOV infection was analyzed. These studies provide further support for the utility of TLR4 agonist, GLA-SE, as a candidate adjuvant for prophylactic protein-based vaccines to prevent EBOV disease.

2. Materials and methods

2.1. Animals, vaccinations, and viral challenge

C57BL/6 mice were obtained from NCI Charles River. Mice between 8 and 12 weeks of age were vaccinated with 100 µl via the intramuscular (IM) route, in the caudal thigh. All mice in each study were female and age-matched and therefore were inherently randomized.

Animals were monitored at least once daily by technical staff members who were blinded to the study aims. Animal status was evaluated according to an Intervention Scoresheet approved by USAMRIID IACUC. The scoresheet provided physical and behavioral characteristics ranging from 0 (Healthy) to 5 (Moribund). Recorded scores of healthy, sick, or moribund were given based on the physical appearance and behavior of the animal. Monitoring increased to three times daily if the animals were given a behavioral and physical appearance score of three or four and animals were recorded as "sick." Animals were euthanized was by CO2 inhalation followed by confirmatory cervical dislocation. Analgesics and anesthetics were not used in this study and animals were euthanized for humane purposes if they reached a score of five or more, which would be indicated if the animals exhibited ruffled fur, weakness, unresponsiveness, and/or difficulty walking. Otherwise, animals were euthanized on day 14 of the study. For all survival studies, control groups included animals vaccinated with saline and/or adjuvant alone.

GLA-SE and GLA-AF was obtained from the Infectious Disease Research Institute, (Seattle Washington) [23]. VLPs were manufactured by Paragon Bioservices and were produced as previously described [5,11]. Briefly, HEK293F cells were transfected with Ebola Zaire virus GP and VP40 genes in pWRG expression vectors, essentially as previously described [24]. VLP were irradiated at 1e6 rad to ensure sterility and contained <25 EU/ml endotoxin and <10 colony forming units of bacteria per vaccination. Vaccines were administered IM two times, with 3 weeks between vaccinations. A challenge dose of 1000 pfu of ma-Ebola virus was administered via the intraperitoneal (IP) route as previously described [25].

2.2. Enzyme-linked immunosorbent (ELISA) assay

Antibody titers were determined using an ELISA. Two µg/ml of recombinant Ebola virus GP was plated in a flat bottom 96 well plate overnight. Plates were incubated with blocking buffer (5% milk, 0.05% Tween in PBS) for 2 h, and then serum samples were added to plates. The standard protocol used half log dilutions starting at a 1:100 dilution. After 2 h, plates were washed with PBS + 0.05% Tween and secondary antibody was added at a 0.6 µg/ml. Secondary antibodies included goat anti-mouse IgG-HRP (Southern Biotech 1030-05), IgG1-HRP (Southern Biotech 1070-05), IgG2c-HRP (Southern Biotech 1079-05), and IgG3-HRP (Southern Biotech 1100-05). One hour later, plates were washed and exposed using Sure Blue TMB 1-component substrate and stop solution (KPL), and the absorbance at 450 nm was recorded. Serum from unvaccinated animals was used to establish background and titers were defined as the serum dilution resulting in an absorbance greater than 0.2, where background was universally <0.2. Serum from animals previously determined to contain anti-GP antibody was included in each assay to serve as a positive control.

2.3. Intracellular Cytokine Staining (ICS)

On study day 25 (4 days post-boost) spleens and popliteal lymph nodes were isolated for T cell assays. After red blood cell lysis, single cell suspensions were cultured at 10e6 cells/ml in complete media (90% RPMI 1640, 10% FBS, 20 mM Hepes, 1% Pen/strep, 0.05 mM BME) with 10 U/ml mouse recombinant IL-2, 1 µg/ml mouse CD49d (BD #553,314), 1 µg/ml mouse CD28 (BD 553295), and 1x protein transport inhibitor cocktail (eBioscience #00-4980). 1e6 cells were plated in each well of a 96 well plate and were stimulated with 2 µg/ml Ebola virus GP peptide WIPYFGPAAEGIYTE (WE15) diluted in DMSO for experimental samples as it had previously been shown to elicit a detectable T cell response in C57BL/6 mice [11,26,27]. Cell stimulation cocktail (eBioscience 00-4970-93) or DMSO were used as a staining control.

Six hours after stimulation, cells were washed in PBS + 10% FBS. Live/Dead aqua (Invitrogen) was used to identify viable cells by incubation for 10 min at 4 °C, and Fc Block (Miltenyi) was used to prevent non-specific antibody binding. After washing, surface antibodies CD3-FITC (BD clone 145-2C11), CD8-APC-H7 (BD clone 53-6.7), CD4-PacBlue (BD clone RM4-5) were incubated with samples for 20 min at 4C and then cells were washed again and fixed with 3.7% paraformaldehyde overnight. Cells were then permeabilized with perm/wash (eBioscience 00-8333-56) and stained with IFNγ-APC antibodies. Cells were run on the Canto II flow cytometer, and analysis was conducted using FlowJo software. Isotype controls and minus one controls were used to define populations. A representative figure illustrating the gating strategy is included in Supplementary Fig. 1.

2.4. ELISPOT assay

MabTech Mouse IFN-γ ELISPOT PLUS kit (3321-2HW-Plus) was used for evaluation of IFN-γ production. Wells of a 96 well-plate were plated overnight with capture antibody, as per the manufacturer's instructions. Spleens and popliteal lymph nodes were isolated from vaccinated animals and subjected to red blood cell lysis. Cells were then resuspended at 2e6/ml and 100 µl of cells was combined with 100 µl of stimulation master mix. Master

mixes included 20 U/ml mouse recombinant IL-2, 2 μg/ml mouse CD49d (BD 553314), 2 μg/ml mouse CD28 (BD 553295), and one of the following stimulants: 4 μg/ml WE15 peptide (WIPYFGPAAEGIYTE, Mimitopes), 0.2 μM GP, or 4 μg/ml DMSO. Cells were incubated in ELISPOT plates for 16 h at 37 °C, and the ELISPOT assay was conducted as per the manufacturer's instructions. Plates were analyzed using the CTL ImmunoSpot reader. Values were calculated by averaging duplicate wells and then subtracting the average of the unstimulated duplicate wells for each animal.

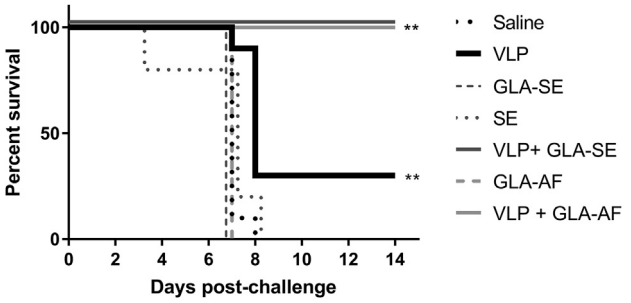

Fig. 1. Inclusion of GLA adjuvants with VLP vaccination results in improved survival after challenge. Animals (n = 10 per group) were vaccinated with Saline (n = 10), 1.25 μg VLP (n = 10), 5 μg GLA-SE adjuvant (n = 10), 5 μg SE adjuvant, 5 μg GLA-AF (n = 10) adjuvant, or 1.25 μg VLP + 5 μg GLA-AF (n = 10), as a homologous prime boost on day 0 and Day 21. Animals were challenged on day 49, four weeks after the vaccine boost, with 1000 pfu of ma-EBOV. Survival was analyzed up to 14 days post-challenge. Kaplan-Meier survival analysis with log-rank comparison was used for statistical analysis, $^{**}p < 0.01$.

2.5. IFN-γ/IL-2 mouse fluorospot

Mice were sacrificed Day 35 after vaccination, or D4 post vaccine boost. Spleen and lymph nodes were collected and T cell responses were measured using the double color IFN-γ/IL-2 FluoroSpot assay per manufacturer's protocol (Cellular Technology Limited, #mT2002F, mT02, mT16). Briefly, special low autofluorescent PVDF filter plates were activated with 70% ethanol before murine IFN-γ and IL-2 capture antibodies were added. Coated plates were incubated overnight. Splenocytes and lymph cells were brought to a concentration of 2e6/ml in serum-free CTL-Test Medium (CTL, #CTLT-005) supplemented with 1% L-glutamine and 10 μg/ml cytokine combo CD28/Cd49d. Cells were tested for reactivity against the peptide WE15 (WIPYFGPAAEGIYTE, Mimitopes). Cell stimulation cocktail (eBioscience, #00-4970-93) utilized at a 1:250 dilution was used a positive control and DMSO as a negative control. After a 24 h incubation period, bound IFN-γ was detected with FITC detection Alexa Fluor 488 antibody and IL-2 with a biotin streptavidin CTL-Red. Fluorescent spots were enumerating using CTL S5 ImmunoSpot Analyzer running FluoroX Suite software.

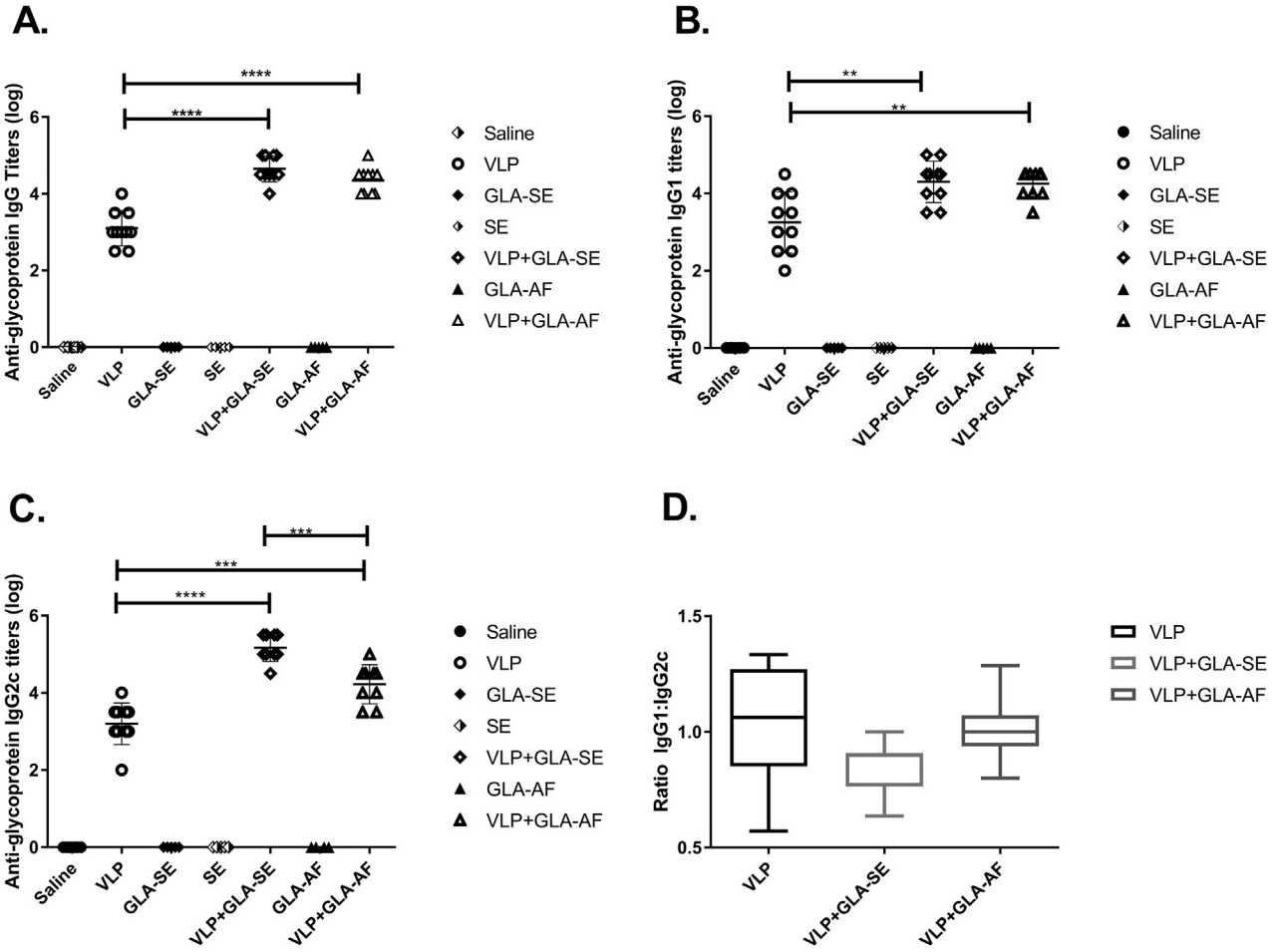

Fig. 2. Anti-GP IgG, IgG1, and IgG2c antibody titers following homologous prime-boost. In the above experiment, serum was collected on day 35, two weeks after vaccine boost, and was evaluated for anti-EBOV glycoprotein (GP) antibody titers. **A**, anti-EBOV GP IgG titers were analyzed by ELISA. **B**, anti-EBOV GP IgG1 titers were analyzed by ELISA. **C**, anti-EBOV GP IgG2c titers were analyzed by ELISA. **D**, the ratio of anti-EBOV GP IgG1 to IgG2c titers is shown. Non-parametric ANOVA (NPAR1WAY) was used for statistical analysis of in A-C. Parametric ANOVA was used for statistical analysis of D. *, $p < 0.05$ and **, $p < 0.01$, ***, $p < 0.001$; and ****, $p < 0.0001$. Error bars represent standard deviation (SD).

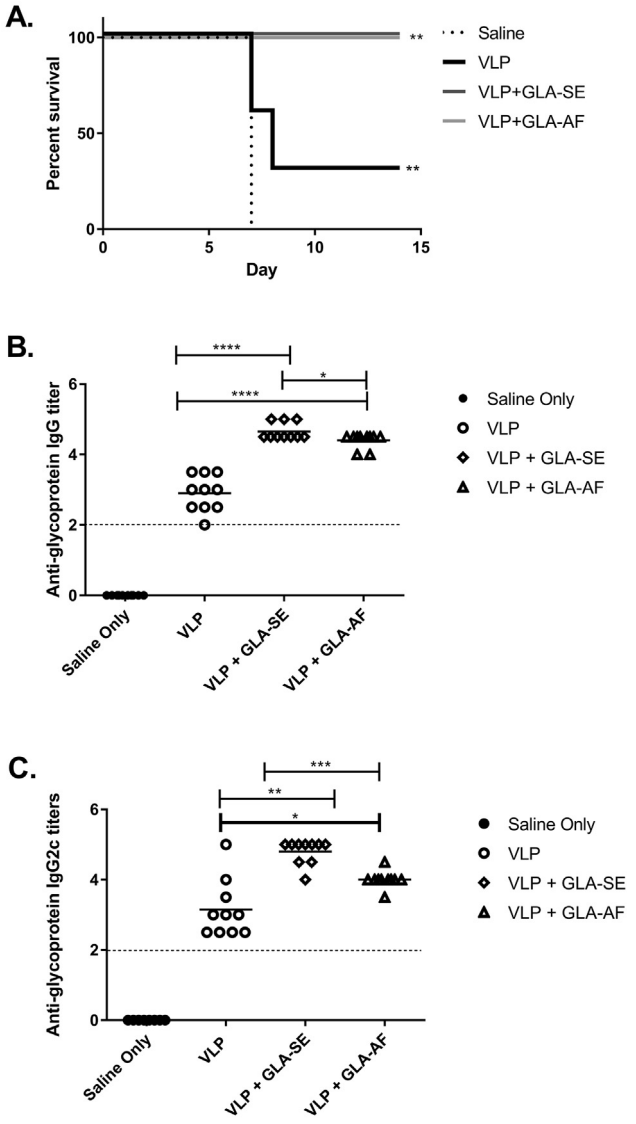

Fig. 3. Anti-GP IgG, IgG1, and IgG2c antibody titers following prime vaccination. Animals were vaccinated with Saline (n = 10), 1.25 μg VLP only (n = 10), 1.25 μg VLP + 5 μg GLA-SE (n = 10), or 1.25 μg VLP or VLP + 5 μg GLA-AF (n = 10). Animals were challenged four weeks post-vaccination with 1000 pfu ma-EBOV. **A**, survival was analyzed fourteen days post-challenge. Kaplan-Meier survival analysis with log-rank comparison was used for statistical analysis. Serum was collected two weeks after vaccination and evaluated for anti-EBOV GP antibody titers. **B**, anti-EBOV GP IgG titers were analyzed by ELISA. **C**, anti-EBOV GP IgG2c titers were analyzed by ELISA. Non-parametric ANOVA (NPAR1WAY) was used for statistical analysis of in A-C. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. Error bars represent SD.

2.6. Statistical analysis

Statistical analyses were performed using SAS Version 9.4 (2012 SAS Institute, Cary, NC). Continuous variables were screened for normality and homogeneity of variance. IgG, IgG1, IgG2c, and, T-cell responses were analyzed using nonparametric methods, ANOVA (NPAR1WAY). In instances where multiple group comparisons using nonparametric methods were required, Kruskal–Wallis tests were initially used followed by the Dwass, Steel, Critchlow-Fligner (DSCF) multiple pairwise comparison procedure to control the familywise error rate. IgG1:IgG2c ratios met assumption of normality and homogeneity of variance and were analyzed using one-way analysis of variance (ANOVA) with post-hoc Tukey's studentized range tests for pairwise comparisons. Percentage surviving between groups were compared using Kaplan-Meier survival analysis with log-rank comparison of survival curves between groups. Stepdown Bonferroni corrections were used to conduct multiple comparisons and control the familywise error rate.

3. Results

3.1. GLA-SE enhances efficacy of VLP vaccination and elicits Th1 skewed antibodies

A dose of ten micrograms of VLP (based on GP content) is protective against challenge with 1000 pfu ma-EBOV when administered prophylactically as a homologous prime-boost (day 0, day 21) and when challenge occurs 4 weeks following booster vaccination [28]. Decreasing the dose by approximately ten-fold to 1.25 μg (sub-optimal dose) consequently decreases survival to 10–30%. Female C57BL/6 mice, age 8–10 weeks, were vaccinated intramuscularly on day 0 and boosted on day 21 with a suboptimal dose of VLP. Five micrograms of GLA adjuvant in a stable emulsion (-SE) or in aqueous formulation (-AF) was combined with the VLP vaccine. Control groups received saline, VLP, or adjuvant alone. Four weeks after the vaccine boost, animals were challenged with 1000 pfu of ma-EBOV. All adjuvant-only animals succumbed to infection on the same schedule as saline-treated animals. As anticipated for the model, 30% of mice vaccinated with VLP alone survived. The addition of 5 μg of either of the GLA adjuvant formulations in combination with VLP resulted in 100% survival after challenge with ma-EBOV (Fig. 1).

Two weeks after the vaccine boost, blood was collected and serum EBOV-specific anti-GP antibody titers were evaluated by ELISA. When given with VLP, GLA-SE and GLA-AF significantly enhanced anti-EBOV GP antibody titers, including overall IgG, IgG1, and IgG2c compared to VLP alone. Adjuvant alone had no impact on anti-GP antibody titers (Fig. 2A-D). The addition of GLA-SE to VLP vaccine resulted in modest increase in IgG titers compared to GLA-AF (Fig. 2A). Significant differences between the adjuvants were more readily observed when examining the IgG isotypes. Previously reported data have shown that enhanced IgG2c, a Th1-skewed isotype which promotes Th1 type T cell immunity, has correlated to protection in the prime-boost model (Fig. 2C) [28]. GLA-SE in combination with VLP induced IgG2c titers that were significantly higher than those induced by VLP alone or VLP in combination with GLA-AF ($p = 0.003$, respectively) (Fig. 2C). The notable increase in IgG2c resulted in a decreased IgG1:IgG2c ratio, supporting the presence of Th1-skewed antibodies induced by the inclusion of GLA-SE to VLP vaccination (Fig. 2D).

3.2. The inclusion of GLA adjuvants with VLP vaccination provides protection with a single vaccination

Given the observed efficacy of inclusion of GLA adjuvants with VLP as a homologous prime boost, we next evaluated the impact of adjuvant on survival with single dose vaccination. Female C57BL/6 mice, age 8–10 weeks, were vaccinated intramuscularly on day 0 and challenged 4 weeks later with 1000 pfu ma-EBOV. As anticipated for this model, vaccination with VLP alone resulted in partial protection, as 30% of the animals survived ma-EBOV challenge. Inclusion of GLA adjuvants, GLA-SE or GLA-AF, with VLP vaccination resulted in complete protection, as 100% mice survived viral challenge (Fig. 3A). Previous studies have shown no efficacy with adjuvant alone in the absence of VLP vaccination (data not shown). These results suggest that either formulation of GLA, GLA-SE or GLA-AF, can enhance efficacy of a single VLP vaccination.

In addition to enhanced vaccine efficacy, the inclusion of GLA adjuvants with VLP also corresponded to improved antibody

responses with a single vaccination compared to vaccination with VLP alone. GLA-SE combined with VLP significantly increased anti-EBOV GP IgG titers compared to all other treatment groups 14 days following vaccination (Fig. 3B). Generally, the inclusion of adjuvants to VLP significantly increased IgG2c titers; the greatest increase in titer was observed in animals that received a combination of GLA-SE and VLP (Fig. 3C). Based on these results, GLA-SE was selected for further evaluation.

3.3. The inclusion of GLA-SE to VLP vaccination results in durable protection against EBOV

GLA-SE in combination with VLP was evaluated in the stringent durable protection model based on the preliminary results showing superior Th1 skewed antibody responses compared to GLA-AF formulation. Mice were vaccinated on day 0 and 21 with 10 μg VLP, with or without inclusion of GLA-SE. Mice were then challenged with 1000 pfu ma-EBOV day 175 (22 weeks post-boost). Mice receiving adjuvant alone or saline alone succumbed to EBOV infection within one week post infection. Vaccination with VLP alone resulted in 10% survival to EBOV challenge. This supports previously published data demonstrating that the VLP alone does not provide lasting protection [28]. Mice receiving VLP vaccination combined with GLA-SE were completely protected from ma-EBOV challenge, indicating that GLA-SE confers durable protection when combined with VLP vaccination (Fig. 4A).

The presence of anti-GP IgG was analyzed on day 35 (2 weeks post-boost). The inclusion of GLA-SE to VLP vaccination resulted in improved anti-GP IgG antibody titers following vaccine boost (Fig. 4B). EBOV GP-specific IgG titers were also assessed one week prior to viral challenge (day 168). The inclusion of GLA-SE adjuvant to VLP vaccination resulted in elevated anti-EBOV GP IgG titers that persisted for 6 months following vaccination (Fig. 4C). Additionally, IgG2c titers were evaluated at the same timepoint and showed that elevated IgG2c titers also persist at this later time point (data not shown).

3.4. GLA-SE enhances Ebola-specific T cell responses

Further immune analyses were carried out to determine if the enhanced Th1 skewed antibodies and improved efficacy observed with the inclusion of GLA-SE with VLP vaccination corresponded to improved effector T cell responses. For these experiments, mice were vaccinated on day 0 and 21. Vaccinations included 10 μg VLP, with or without inclusion of GLA-SE to evaluate the early immune responses corresponding to durable protection. Control groups received saline or adjuvant alone. EBOV GP-specific T cell responses were evaluated in the splenocytes and lymphocytes 4 days following vaccine boost.

VLP alone elicited minimal IFN-γ production in splenocytes as previously described [11] (Fig. 5A). The addition of GLA-SE adjuvant enhanced the production of EBOV GP-specific IFNγ production by immune cells in the spleen (Fig. 5A). Specifically, EBOV-specific IFN-γ production was significantly increased in activated (CD44high) effector CD4+ T cells (Fig. 5C). Adjuvant inclusion also augmented activation of (CD44high) CD8+ effector T cells as

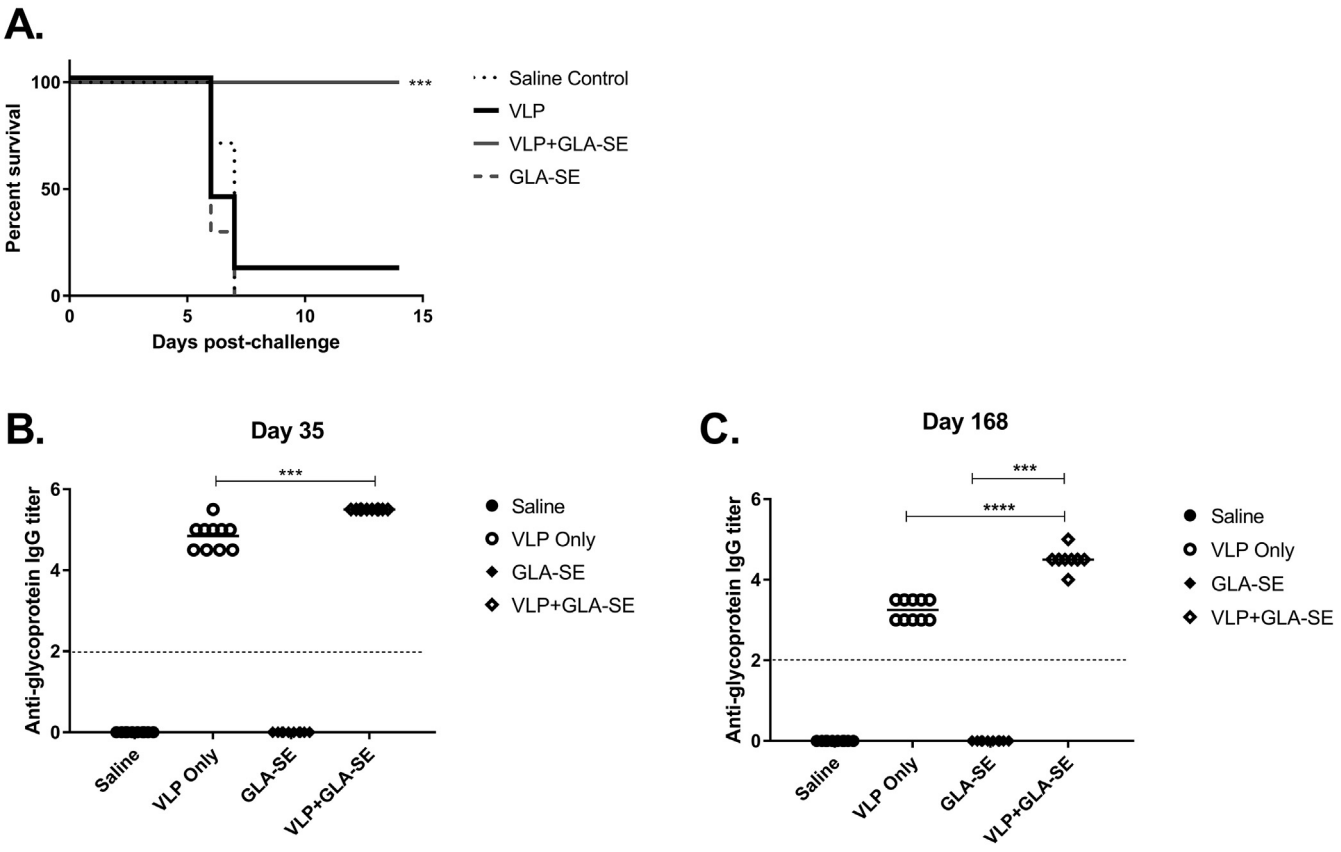

Fig. 4. Survival after ma-EBOV challenge on day 175 and anti-EBOV GP antibody titers on day 35 and day 168 following vaccination. Animals were vaccinated with Saline (n = 10), 10 μg VLP only (n = 10), 10 μg VLP + 5 μg GLA (n = 10) or 5 μg GLA only (n = 10) as a homologous prime boost on day 0 and Day 21. Animals were challenged on day 175 (22 weeks post-vaccine boost) with 1000 pfu ma-EBOV. **A,** survival was analyzed up to 14 days post-challenge. Kaplan-Meier survival analysis with log-rank comparison was used for statistical analysis. Serum was collected two weeks after vaccine boost (day 35) and one week prior to challenge (day 168). **B**, day 35 sera was evaluated for anti-EBOV GP IgG titers by ELISA **C**, day 168 sera was evaluated for anti-EBOV GP IgG titers by ELISA. Nonparametric ANOVA (NPAR1WAY) was used for statistical analysis for B-C. $^{***}p < 0.001$ and $^{****}p < 0.0001$. Error bars represent SD.

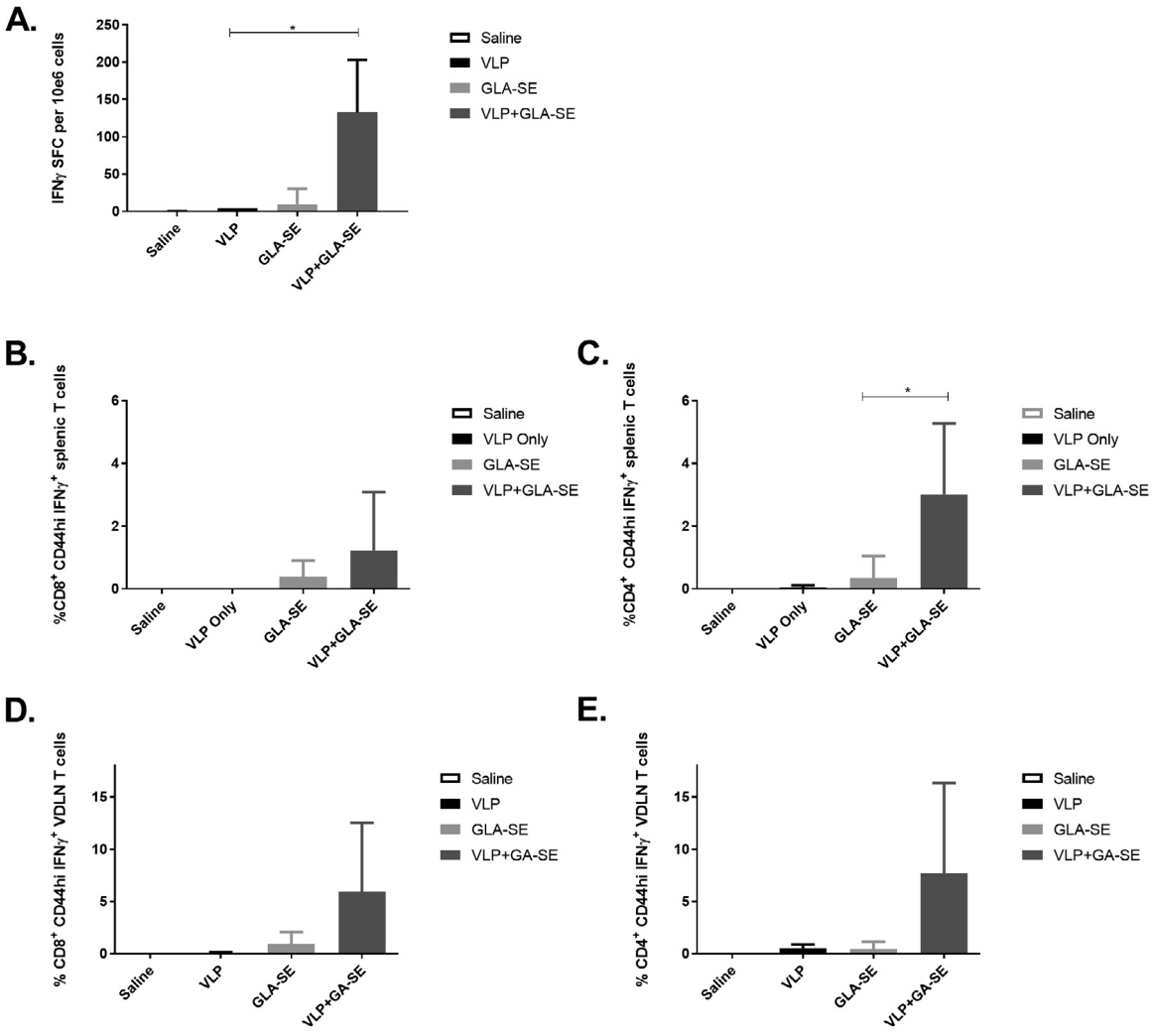

Fig. 5. GP-specific T cell IFNγ responses from spleen and vaccine-draining lymph nodes. Mice were vaccinated on day 0 and day 21 with Saline (n = 5), 10 μg VLP only (n = 5), 10 μg VLP + GLA-SE (n = 5) and GLA-SE (n = 5). Spleens and vaccine-draining lymph nodes (VDLN), specifically popliteal lymph nodes, were harvested four days following boost. **A**, EBOV GP-specific IFNγ production in splenocytes was assessed by ELISPOT by measuring reactivity to WE15 peptide stimulation. Spleens and VDLN were pooled by treatment group. **B**, GP-specific CD44high effector CD8+ cells producing IFNg in response to WE15 peptide stimulation were analyzed in the spleen. **C**, CD4+ cells producing IFNg in response to WE15 peptide stimulation were analyzed in the spleen. **D**, GP-specific CD44high effector CD8+ cells producing IFNg in response to WE15 peptide stimulation were analyzed in the VDLNs by ICS. **E**, CD4+ cells producing IFNg in response to WE15 peptide stimulation were analyzed in the VDLNs by ICS. Nonparametric ANOVA (NPAR1WAY) was used for statistical analysis; $^{*}p < 0.05$. Error bars represent SD.

measured by IFN-γ secretion (Fig. 5B). IFN-γ production by T cells in the vaccine-draining lymph node (VDLN) also increased as a result of the inclusion of GLA-SE to VLP vaccination (Fig. 5D and E).

In a separate experiment, mice were vaccinated on day 0 and 21 with 10 μg VLP, with or without inclusion of GLA-SE. Control groups received saline or adjuvant alone. In these experiments, SE alone was included as a control to delineate the immune responses elicited by GLA versus those by the SE adjuvant vehicle. Spleens were harvested 4 days following vaccine boost and analyzed for IL-2 and IFN-γ production after EBOV GP-specific, WE15 peptide stimulation by fluorescent ELISPOT (Fluoro-SPOT). The analysis by Flouro-SPOT allowed for the enumeration of single cytokine secreting cells as well as cells secreting dual cytokines. The addition of GLA-SE enhanced the production of EBOV GP-specific IFN-γ and IL-2 production compared to vaccination with VLP alone in VDLNs and spleen (Fig. 6A, B, D, E). SE alone also increased the production of IL-2, however unlike GLA-SE, it did not result in the induction of IFN-γ secreting cells. GLA-SE inclusion with VLP resulted in a significant increase in the number of cells secreting both IL-2 and IFN-γ. These data support the data from the previous experiment indicating that GLA-SE increases the magnitude of the activated effector T cells in response to vaccination. Taken together with the ICS and ELISPOT data, the presence of GLA-SE augments the activation of polyfunctional effector T cells that play a role in the induction of durable immunity and promote long-term protection against ma-EBOV.

4. Discussion

The nature of the immune response required to provide protection against Ebola virus is undefined. Evidence suggests that the efficacy of different vaccine platforms may vary depending on the vaccine platform: while antibody is the primary immune correlate of the rVSV-ZEBOV vaccine, CD8 T cell responses have been attributed to protection by the Ch-Ad5 platform [29,30]. Previous studies conducted in animal models, including mice and non-human primates, have shown that vaccination with EBOV VLP is efficacious against lethal Ebola challenge. However addition

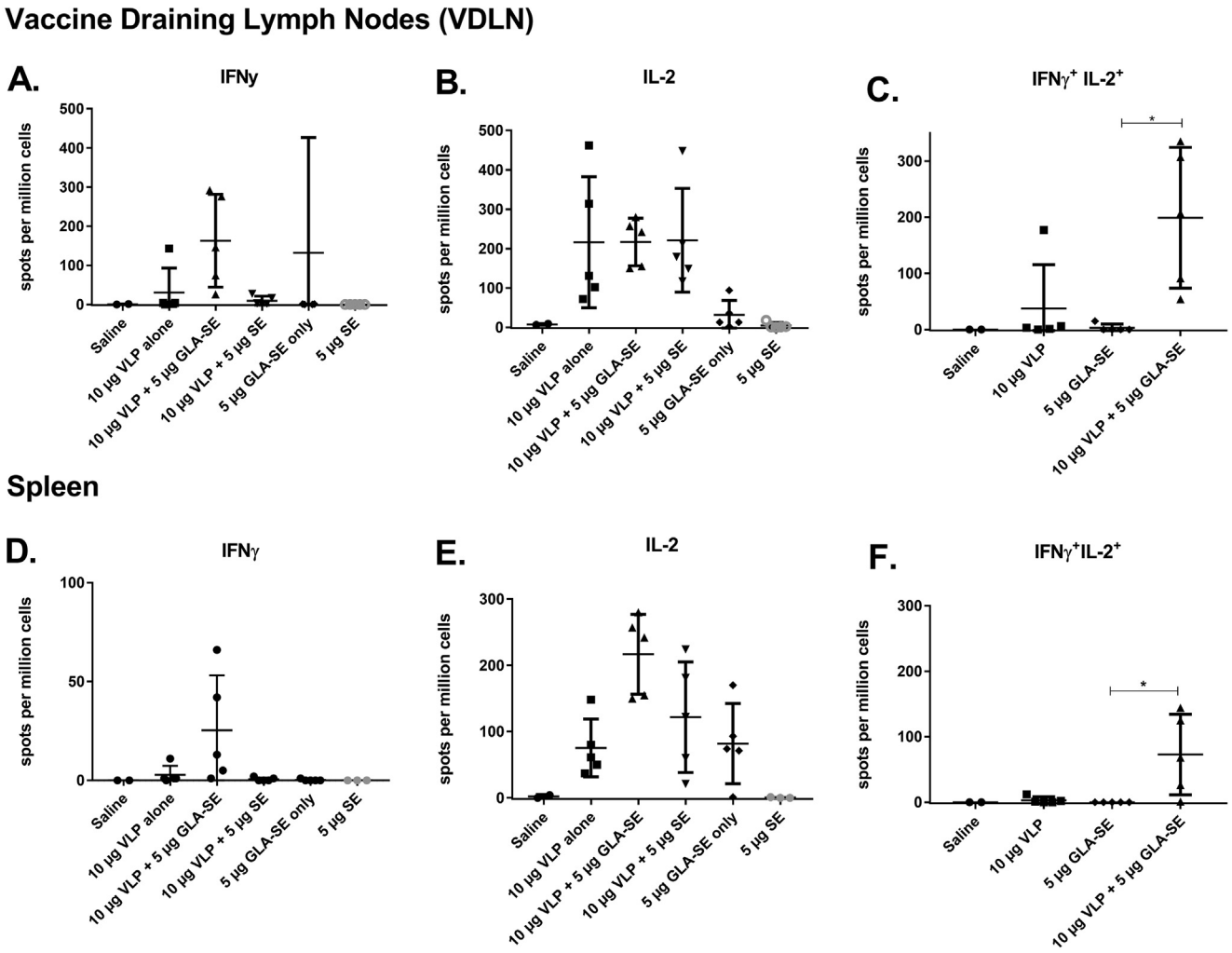

Fig. 6. The inclusion of GLA-SE and VLP increased poly-functional T cells. Mice were vaccinated on day 0 and day 21 with Saline (n = 5), 10 μg VLP only (n = 5), 10 μg VLP+ GLA-SE (n = 5), GLA-SE (n = 5), and SE (n = 5). Spleens and vaccine-draining lymph nodes (VDLN), specifically popliteal lymph nodes, were harvested four days following boost and analyzed for GP-specific IL-2 and IFNγ secreting cells by measuring reactivity to WE15 peptide stimulation by Florospot. **A**, Cells producing GP-specific IFNγ were enumerated in the VDLNs. **B**, Cells producing GP-specific IL-2 were enumerated in the VDLNs. **C**, Cells producing both GP-specific IFNγ and IL-2 were enumerated in the VDLNs. **D**, Cells producing GP-specific IFNγ were enumerated in the spleen. **E**, Cells producing GP-specific IL-2 were enumerated in the spleen. **F**, Cells producing both GP-specific IFNγ and IL-2 were enumerated in the spleen. Nonparametric ANOVA (NPAR1WAY) was used for statistical analysis; *, $p < 0.05$. Error bars represent SD.

of adjuvants, such as QS21, RIBI, and Poly-IC-LC [6,8,11], can provide dose-sparing and enhance vaccine-induced immune responses. The work described here aims to characterize the immune response associated with one effective adjuvant, GLA-SE.

Protection against Ebola virus has been reported to be accompanied by the generation of vaccine-induced anti-GP antibodies [31]. With one adjuvant, improved protection after vaccination was linked to Th1-biased immune responses [28]. Specifically CD4+ T cells appeared to play a critical role in promoting lasting protection from EBOV challenge [28,32]. Understanding the link between protection and specific immune modulation should guide the selection of adjuvants that elicit the appropriate immune responses to drive lasting immunity and protection.

The TLR4 agonist, GLA-SE, has been being tested in multiple preclinical and clinical trials as a vaccine adjuvant and has shown safety and efficacy in both animals and humans [23,33–35]. It has been reported that the GLA-SE enhanced functional vaccine-induced antibody responses and induced Th1 immunity, specifically through the activation of CD4+ IFN-γ producing T cells [22,36–40]. Additionally, the benefit of the SE formulation of GLA over other formulations, including AF, has been the ability to robustly augment Th1 immunity, in part, through enhancing the magnitude of Th1 skewed antibodies [23,41].

In our studies, we have leveraged the EBOV VLP vaccine model to analyze the efficacy of GLA adjuvants to rescue survival when administered with a sub-optimal dose of VLP [11]. GLA-SE combined with EBOV VLP completely restored survivability, thus demonstrating its efficacy and dose-sparing effects [11]. Additionally, complete protection against EBOV challenge was seen after 22 weeks following booster vaccination, demonstrating the establishment of durable protection.

The addition of GLA-SE to VLP vaccination enhanced Ebola-specific anti-GP antibody titers. Notably, adjuvant inclusion lowered the ratio of Th2:Th1 skewed antibodies to promote the enhancement of Th1 skewed IgG isotype, IgG2c. This occurred even without the requirement of a booster vaccination. Additionally, high titers of EBOV-specific IgG persisted 22 weeks following vaccination. Thus, GLA-SE promotes rapid production of antigen-specific antibodies that persist to promote durable immunity to EBOV.

The inclusion of GLA-SE enhanced CD4+ and CD8+ T cell responses. T cell responses were largely undetectable in animals

vaccinated with VLP alone, but the addition of GLA-SE resulted in antigen-specific, effector CD8+ and CD4+ T cells. GLA-SE has been reported to stimulate the production of IFN-γ which may increase cross-presentation of antigen resulting in enhanced CD8+ T cell activation and increased efficacy against viral pathogens [47]. Also, the enhanced effector function of Th1 helper CD4+ T cells may aid in sustaining the effector CD8+ T cell population. When antigenic protein is presented in the absence of TLR4 stimulation, as occurs when SE adjuvant is administered alone, cells may be signaled to proliferate but are not promoting Th1 cell differentiation, as indicated by the presence of IL-2 and the absence of IFN-γ production. Additionally, elevated local IL-2 can recruit regulatory CD4+ T cells (Tregs) which may dampen the desired inflammatory response and blunt the establishment of protective immunity. This demonstrates the importance of combining an immunostimulatory adjuvant, such as GLA, with a dispersal adjuvant, SE, to induce the appropriate anti-viral T cell response.

Studies have reported that multiple cytokine-producing cells have enhanced anti-viral effector function compared to single cytokine-producing cells [42]. Thus, polyfunctional T cell responses are associated with effective vaccination in several vaccine models and are thought to be required for antiviral immunity [28,30,32,43–46]. These findings support that activating the innate immune receptor TLR4 during vaccination augments adaptive immune responses to yield superior anti-EBOV effector multi-cytokine producing T cells.

Cumulatively, these data show that TLR4 activation with GLA-SE at the time of vaccination promotes dose sparing effects, induces robust, persistent antibody production and enhances EBOV-specific T cell responses, leading to long-lasting protective immunity. This study extends the EBOV VLP as a vaccine platform which can be partnered with GLA-SE, showing efficacy as a prime vaccination as well as promoting long term efficacy.

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Compliance with ethics guidelines

Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The IACUC committee approving this protocol is the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) IACUC. The facility where this research was conducted, USAMRIID, is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the 8th Edition of the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Author contributions

Conceived and designed the experiments: MMES and KAOM. Performed the experiments: MMES, MG, JTS, and SVT. Analyzed the data: MMES and KAOM and JST. Contributed reagents/materials: NVH, Performed statistical analysis of manuscript data: PGB. Wrote the manuscript: MMES and SB.

Declaration of Competing Interest

The authors have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.05.026.

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