Adjuvant-enhanced CD4 T Cell Responses are Critical to Durable Vaccine Immunity

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1. Introduction

Traditional vaccine development has focused on highly immunogenic live-attenuated or inactivated pathogen platforms. However, safety concerns associated with these platforms, as well as advances in vaccine manufacturing and antigen characterization, have turned interest toward protein-based vaccines. Protein-based vaccines are designed to elicit an immune response against a specific antigen with known protective capabilities. While they are a safer alternative to traditional vaccines, they are also less immunogenic and confer less durable immune protective capabilities. In this study, four adjuvants were tested in combination with the Ebola virus VLP vaccine to determine their impact on durable protection. We demonstrate that the most effective adjuvant elicits a Th1-skewed antibody response and strong CD4 T cell responses, including an increase in Tfh frequency. Using immune proficient animals and adoptive transfer of serum and cells from vaccinated animals into naïve animals, we further demonstrate that serum and CD4 T cells play a critical role in conferring protective within effective vaccination regimens. These studies inform on the requirements of long term immune protection, which can potentially be used to guide screening of clinical-grade adjuvants for vaccine clinical development.

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resulting in prolonged antigen exposure, which may or may not contribute to adjuvancy (Hutchison et al., 2012). Additionally, alhydrogel has been shown to activate the inflammasome, which may contribute to the immunogenicity of alhydrogel-based vaccines (Guven et al., 2013; Gupta, 1998; Hogeness, 2002; Marrack et al., 2009). PolyICLC is a double-strand RNA stabilized by poly-L-lysine in carboxymethylcellulose (Levy et al., 1975). It signals through TLR3 and potentially MDAS receptors, eliciting a strong type I IFN response, and it skews the immune response toward a Th1 profile response (Wang et al., 2010; Alexopoulos et al., 2001; Nemes et al., 1969). PolyICLC has been in multiple clinical trials for both therapeutic and vaccine purposes (Martins et al., 2015b).

There are three different classes of CpG molecules, which target different cell subsets and receptors and have different recognition in mouse and human cells (Verthelyi et al., 2001; Hartmann et al., 2003; Marshall et al., 2003). The CpG examined here is a class C CpG (2395), meaning that it signals through both pDC and B cells, impacting type I IFN production, antigen-presenting cell (APC) maturation, and NK cell activation (Marshall et al., 2003). CpG molecules (specifically 7909) have been in multiple clinical trials as vaccine adjuvants; the specific CpG tested in this work has not been in clinical trials but was selected as it targets both human and murine TLR9 and it has both Class A and B activation characteristics. Finally, MPLA is a TLR4 agonist, which is comparable to MPL, the active component of the GSK adjuvant AS04 (Einstein et al., 2014a, 2014b). MPLA has been shown to be highly effective as an adjuvant, particularly in combination with an aluminum-based adjuvant like alhydrogel or a nanoparticle formulation (Bohannon et al., 2013).

Considerable work has gone into evaluating the impact of putative adjuvants on innate immune activation and on adaptive immune responses to model antigens and potential vaccines (Longhi et al., 2009; Kastenmuller et al., 2012; Trumpftheller et al., 2008; Stahl-Hennig et al., 2009; Perrer et al., 2013; Caproni et al., 2012). It has been demonstrated that co-administration of adjuvant and antigen can be critical for optimizing the immune response, and formulation of adjuvants and antigen in nanoparticles for co-administration is currently being explored as a means of targeting the adjuvant effects (Quinn et al., 2013b; Hanson et al., 2015; Moon et al., 2012; Jain et al., 2011). However, adjuvants’ impact on long-lasting protective immunity is poorly understood, particularly in the context of a relevant challenge model (Seder et al., 2015).

The filovirus VLP vaccine has demonstrated efficacy in the murine, guinea pig, and nonhuman primate models of filovirus infection (Swenson et al., 2005, 2008a, 2008b; Warfield et al., 2003, 2004, 2007; Martins et al., 2014, 2015a). To evaluate durable protection, we developed a rigorous murine model for Ebola virus challenge. We then tested the ability of the aforementioned adjuvants to augment protection under this model. Correlates of durable protection were identified by comparing and contrasting immune parameters associated with different levels of protection. These data inform broadly on the impact of classic TLR agonists to enhance the durable protection of a protein-based vaccine, and they demonstrate that adjuvant selection can determine the quality and utility of a vaccine candidate.

2. Methods

2.1. Ethics Statement

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.

2.2. 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. For studies involving CD8-deficient animals (Jackson Laboratory strain 002665) and CD4-deficient animals (Jackson Laboratory strain 002663), C57BL/6j (Jackson Laboratory strain 000664) mice were used as controls.

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. Monitoring increased to three times daily if the animals were given a score of three or four. Euthanization 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.

MPLA (MPLA-SM; extracted from LPS produced by Salmonella minnesota R595) and CpG (CpG 2395 Class C, vac-2395-1; 5′-tgcgtgtttcgcgcgcgc-3′) were from Invivogen and polyICLC (Hiltonol) was provided by Oncovir, Inc.; these adjuvants were diluted with sterile saline after resuspension in DMSO (MPLA) or water (CpG). Alhydrogel was from Brenntag (CAS #21645-51-2, 10 mg/ml) and was provided by Paragon Bioservices and were produced by transfecting HEK293F cells with Ebola Zaire virus GP and VP40 genes in pWRG expression vectors, essentially as previously described (Swenson et al., 2004). VLP were irradiated at 160 rad to ensure sterility and contained less than 25 EU/ml endotoxin and less than 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 mouse-adapted (ma-) Ebola virus was administered via the intraperitoneal (IP) route (Bray et al., 1998). The mouse model of Ebola virus challenge is a well-documented small animal model of Ebola virus challenge and recapitulates some of the symptoms of human Ebola virus infection. It has been used to evaluate multiple vaccines and therapeutics developed against filoviruses.

2.3. Adoptive Transfer Studies

C57BL/6 mice were vaccinated twice with three weeks between vaccinations. Four weeks after the second vaccination, serum and splenocytes were harvested. Negatively selected (“untouched”) T cells (Miltenyi Biotech, 130-095-130), CD4 T cells (Miltenyi Biotech, 130-104-454), or CD8 T cells (Miltenyi Biotech, 130-104-075) were isolated using magnetic separation in accordance with the manufacturer’s instructions. Cell purity was universally greater than 90% and on average 94%. Cells and serum were combined prior to injection into recipient mice. Twenty-four hours after transfer, mice were challenged IP with 1000 pfu of ma-EBOV.

2.4. Antibody Assays

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 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-
overnight. Cells were then permeabilized with perm/wash (eBioscience)
ed from C57BL/6 mice and subjected to red blood cell lysis. Untouched T
cells were used to de
ducted using FlowJo software. Isotype controls and minus one controls
(90% RPMI 1640, 10% FBS, 20 mM Hepes, 1% Pen/strep, 0.05 mM BME)
and subjected to intracellular cytokine staining, as described above.

2.7. T Cell Assays — Sorting of T Cell Memory Populations

Four weeks after the second vaccination, splenocytes were harvest-
ed from C57BL/6 mice and subjected to red blood cell lysis. Untouched T
cells were isolated by magnetic bead separation, in accordance with the
manufacturer's instructions (Miltenyi Biotech, 130-095-130). Cells were
stained with CD44 (BD Clone IM7) and CD62L. (L-Selectin, BD
Clone MEL-14) to identify central and effector memory cell populations,
as well as Live/Dead aqua (Invitrogen) (Henao-Tamayo et al., 2010;
Hikono et al., 2007; Lefrancois and Masopust, 2002; Krishnan et al.,
2007). Cells were then washed two times and sorted on the BD Aria II.
Purity of sorted populations was confirmed on the BD Canto II and cell
purity was universally greater than 95% for each T cell sub-population
added to culture.

Feeder cells were isolated from splenocytes of naive C57BL/6 mice.
CD3+ T cells were depleted using magnetic bead isolation (Miltenyi
Biotech 130-094-973) in accordance with the manufacturer's instruc-
tions. Purity was confirmed on the BD Canto II and cells were universally
greater than 95% CD3 negative.

Feeder cells were plated at 5e5 cells/well in 100 μl of media with
20 U/ml mouse recombinant IL2, 2 μg/ml mouse CD49d (BD Clone
9C10), 2 μg/ml mouse CD28 (BD Clone 37.51), and 0.2 μM GP. Sorted T
cells were added at 1e5–2e5 cells/well in 100 μl. Cells were cultured for
four days; on the third day, 50 μl/well of media with 40 U/ml of IL2
was added to each well. On the fourth day of culture, cells were pelleted
and subjected to intracellular cytokine staining, as described above.

2.8. ELISPOT Assay

MabTech Mouse IFNγ ELISPOT PLUS kit (3321-2HW-Plus) was used
for evaluation of IFNγ production. Cells were pre-plated overnight with
capture antibody, as per the manufacturer's instructions. Splenocytes
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 IL2, 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, 0.2 μM GP, or 4 μg/ml DMSO. Cells were incubat-
ed 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.

2.9. Popliteal Lymph Node Isolation for Transcriptomic Analysis

Popliteal lymph nodes (LN) were isolated from vaccinated mice
seven days after vaccination. LN were filtered on a 70 μm filter and re-
suspended in PBS. Untouched T cells were isolated by magnetic bead
separation, in accordance with the manufacturer’s instructions
(Miltenyi Biotech, 130-095-130). Cells were then lysed with buffer
RLT and RNA was isolated using the Qia gen RNeasy Mini Kit, according
to the manufacturer’s instructions (Qia gen 74104); RNA from 3 to 4 an-
imals per vaccination group was pooled and analyzed using the
SABiosciences PCR Array (T cell and B cell activation array, PAMM-
0532) on an ABI 7900 HT real time PCR instrument, in triplicate. Ct
values were normalized to housekeeping genes and the fold difference
in expression of genes in the T cells of animals vaccinated with VLP
and polyICLC vs. VLP alone was determined.

2.10. Popliteal Lymph Node Isolation and Identification of T Follicular Helper Cell Populations

Popliteal LN were isolated from vaccinated mice 7 days after vacci-
nation. LN were filtered on a 70 μm filter and resuspended in PBS.
Cells were plated in 96 well plates and viable cells were identified using
Invitrogen’s Live/Dead aqua dye. T follicular helper (Tfh) cell pop-
ulations were identified by surface staining with CD3, CD4, CXCR5, ICOS,
and PD1 as well as intracellular staining for Bcl6.
CXCR5 was identified by incubating cells with purified rat anti-mouse CXCR5 (BD Clone 2G8) at 1:100 in buffer A (PBS + 0.5% BSA + 0.1% sodium azide supplemented with 2% normal mouse serum (NMS) and 2% FCS) for 1 h. Cells were then washed two times and incubated with goat anti-rat (H + L)-biotin (Jackson ImmunoResearch, 112-067-003) in buffer A for 30 min. Cells were then washed twice and incubated with streptavidin-PECy7 (BD 557598), anti-CD3-V450 (BD Clone 500A2), anti-CD4-PerCyCy5.5 (BD Clone RM4-5), anti-CD1-APC (eBioscience Clone RMPI-30), and anti-ICOS-PE (BD Clone 7E17G9) in buffer A for a further 30 min. After two more washes, cells were incubated with BD Phosflow Lyse/Fix buffer (BD 558049) for 12 min at 37 °C, washed two times, and incubated with anti-Bcl6-Alexa488 (BD Clone K112-91) for 1 h in Perm/Wash Buffer I (BD 557885). Cells were washed two times and examined on the BD FACS Canto II.

2.11. 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, IgG3, T-cell responses, cytokines, chemokines, and splenocytes were analyzed by nonparametric methods. In instances where multiple group comparisons using non-parametric 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. In instances where only two-sample comparisons were required, Mann-Whitney–Wilcoxon rank-sum tests were used, and, where required, stepdown Bonferroni corrections were used to control the familywise error rate. Neutralizing antibody titers 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 was compared using Fisher’s exact tests. In instances where multiple pairwise survival comparisons were required, stepdown Bonferroni corrections were used to control the familywise error rate. Cochran-Armitage test for trend was used to evaluate survival across groups with increasing time to challenge. For IgG and IgG1, values below the lower limits of detection (LOD) (100) were set to a value equal to the LOD divided by the square root of 2 (100/√2). For IgG2c and IgG3, values below the lower limits of detection (LOD) (10) were set to a value equal to the LOD divided by the square root of 2 (10/√2).

3. Results

3.1. Adjuvants can Enhance the Duration of Vaccine-Mediated Protection

We previously demonstrated that VLP expressing GP and VP40 of Ebola Zaire can confer protection from ma-EBOV challenge in C57BL/6 mice. Inclusion of adjuvant provided vaccine dose-sparing, but the durability of the protective immune response was unclear (Martins et al., 2014). To test the efficacy of VLP vaccination in a durable challenge model, we vaccinated mice two times with 10 μg of VLP, a dose level that is protective when challenge occurs 4 weeks after vaccine boost. We then challenged the animals 22 weeks after the vaccine boost. One hundred percent of animals challenged on the short-term schedule survived challenge, as anticipated; however, only 37.5% (25/66) of animals challenged 22 weeks after vaccination survived (Fig. 1a) (p = 0.0001). Using a serial challenge strategy, we vaccinated animals as before and then challenged 8 weeks, 12 weeks, 18 weeks, or 22 weeks after the second vaccination. We observed that protection declined as the length of time from vaccination to challenge increased (Fig. 1b) (p = 0.0046). This decline was concurrent with a drop in antibody titer, and significant differences between challenge days, regardless of survival status, were found for IgG (p = 0.0002), IgG1 (p = 0.0005), and IgG2c (p = 0.0009). The sample sizes were insufficient to compare survivors and non-survivors within each challenge day, however (Fig. 1c).

We next evaluated whether inclusion of adjuvants could augment durable protection. Using data from short-term challenges, we identified the dose level of adjuvant that could provide dose-sparing on the short-term schedule. We then evaluated the efficacy of that dose level

Fig. 1. Adjuvants impact the durability of protection conferred by VLP vaccination. (a) C57BL/6 mice were vaccinated IM two times with 10 μg of eVLP and challenged four (short-term) weeks or twenty-two (long-term) weeks after the vaccine boost. Data in A are pooled from 8 individual studies with 6–10 animals/group. Fisher’s exact test: survival in the short-term group was significantly higher than in the long-term group (p < 0.0001). (b) C57BL/6 mice were vaccinated IM two times with 10 μg of eVLP and challenged at the indicated days after the second vaccination, n = 9 or 10/group. Cochran-Armitage test: percentage surviving declined as time to challenge increased (p = 0.0046). There was a significant difference (p = 0.03) between survival on Day 77 and Day 175. (c) Serum samples collected from animals in (b) one week prior to challenge were subjected to an ELISA for the evaluation of anti-GP IgG, IgG1, and IgG2c antibody titers. Red symbols indicate titers of animals that succumbed to challenge while black indicate titers of survivors; red symbols with black outlines indicate that one of these two animals succumbed to challenge, but animal tags were indeterminate after challenge. Median and IQR shown. (d) C57BL/6 mice were vaccinated two times (IM) with VLP, with or without the indicated adjuvants. Animals were challenged twenty-two weeks after the vaccine boost. Data in D are pooled from at least 4 separate studies with a total of at least 35 animals per group. P-values comparing VLP alone to vaccination with VLP and adjuvant are shown, calculated using Fisher’s exact tests with stepdown Bonferroni correction. V = VLP, VP = VLP + PolyICLC, VC = VLP + CpG, VM = VLP + MPLA, and VA = VLP + alhydrogel.
on VLP vaccination under the durable protection model. C57BL/6 mice were vaccinated with 10 μg of VLP alone or in combination with 10 μg of polyICLC, MPLA, CpG ODN, or 10% alhydrogel. As shown in Fig. 1d, inclusion of polyICLC rescued VLP-mediated durable protection, resulting in 100% survival, and this was a significant increase compared to survival after vaccination with VLP alone (p < 0.0001). Inclusion of CpG resulted in an increase in survival compared to VLP alone, but this was not significant (p = 0.179). Inclusion of MPLA or alhydrogel had no impact on survival. These data provided a clear basis by which effective and ineffective vaccination regimens could be evaluated, permitting the identification of correlates of immune protection.

3.2. Adjuvants Associated with Durable Protection Elicit a Th1 IgG Response

Antigen-specific IgG titers are frequently used as a correlate of vaccine-mediated protection, including in some EBOV challenge models (Wong et al., 2012). The efficacy of the rVSV-ZEBOV vaccine appears to be highly dependent upon antibody, for example, and anti-GP antibodies have been used successfully as therapeutics in NHP models of infection (Wong et al., 2012, 2014; Qu et al., 2012; Dye et al., 2012). In the present study, all four adjuvants significantly enhanced anti-GP IgG antibody titers by Day 35 when compared to vaccination with VLP alone, with p-values less than 0.0017 (Fig. 2a,b). By Day 168, 1 week prior to challenge, titers after vaccination with polyICLC, MPLA, or alhydrogel were still significantly higher than titers after vaccination with VLP alone (p < 0.04), but titers from animals vaccinated with VLP and CpG were comparable to those of animals vaccinated with VLP alone. The subclass of IgG significantly impacts the function of the antibody. In C57BL/6 mice, a Th2 skewed immune response is marked by higher IgG2c titers while a Th1 skewed immune response is marked by high IgG2c and IgG3 titers; these isotypes were therefore selected for analysis (Finkelman et al., 1988; Snapper and Paul, 1987). There were many statistically significant differences between vaccination groups (Fig. 2b), and the overall trends are presented in Fig. 2c. Mice vaccinated with VLP and alhydrogel had significantly higher IgG1 titers than mice vaccinated VLP alone or VLP with CpG, and this was true at both time points. In contrast, these same mice had the lowest IgG2c and IgG3 titers, in keeping with the published observation that alhydrogel elicits a Th2 skewed immune response. In contrast, animals vaccinated with VLP and polyICLC, the vaccination regimen that conferred protection, had the highest IgG2c titers of all groups at both time points (p < 0.015).

In order to determine whether antibody neutralization was impacted by the inclusion of adjuvants, we evaluated neutralization using the PsVNA, which measures neutralization of pseudoparticles expressing Ebola GP. At the early time point, serum from animals vaccinated with VLP and polyICLC had significantly higher NAb titers as compared to VLP alone or VLP administered with any other adjuvant (p < 0.0001) (Fig. 2d). At the late time point, vaccination with VLP and alhydrogel induced the highest NAb titers on average, despite the fact that alhydrogel had no beneficial impact on survival. Titers in animals vaccinated with VLP and alhydrogel were significantly higher than those of VLP with MPL or CpG on day 168 (p < 0.04).

3.3. Adjuvants Associated with Durable Protection Elicit Antigen-Specific CD4 and CD8 T Cell Responses

VLP-mediated protection from Ebola virus challenge has been associated with the presence of antigen-specific T cell responses as well as antibody. To evaluate the impact of the adjuvants on overall IFNγ-producing CD4 T cell responses, we euthanized mice 4 days after the vaccine boost and evaluated IFNγ production via ELISPOT analysis. For the antigen, we utilized a known CD4 and CD8 T cell epitope, the peptide WE15, as described in the Methods section (Fig. 3a). Additionally, we used full-length recombinant GP as the antigen in a duplicate set of experiments, and we observed the same pattern of IFNγ production regardless of the antigen used (Fig. 3a and Supplemental Fig. 1).

ELISPOT data indicated that polyICLC induced the highest frequency of IFNγ-producing cells, which was significantly higher than the frequency of cells elicted by VLP vaccination alone or with any other adjuvants (p < 0.04) (Fig. 3a). Vaccination with CpG and VLP elicited the second highest IFNγ response, which was approximately half that observed with VLP and polyICLC. To determine whether the response observed after vaccination with VLP and polyICLC or CpG was CD4 or CD8 T cell mediated, we performed intracellular cytokine staining on splenocytes on day 4 after the vaccine boost. Cells from animals vaccinated with VLP and polyICLC or VLP and CpG were evaluated and, in keeping with the ELISPOT results, we observed that polyICLC elicited higher frequencies of antigen-specific IFNγ-producing cells than CpG, though this was not significant, as well as higher frequencies of TNFα-producing CD4 T cells (p = 0.0209) (Fig. 3b,c).

In order to evaluate the persistence of this T cell response, we vaccinated mice two times, and then we left them to rest for 22 weeks. At that time, when we would normally challenge for the durable challenge model, we administered a third vaccine boost and then euthanized animals 4 days later. We performed ELISPOT analysis on splenocytes from these animals and observed, as anticipated, a lower overall frequency of IFNγ-producing cells as compared to Day 4 after the boost (Fig. 3d). However, animals vaccinated with VLP and polyICLC still had significantly higher frequencies of antigen-specific cells as compared to animals vaccinated with VLP alone or VLP with alhydrogel (p < 0.05).

We next hypothesized that the inclusion of adjuvants may impact not only the frequency of antigen-specific T cells, but also the memory phenotype. The frequency of antigen-specific T cells in vaccinated mice is quite low after the peak response. We therefore developed an assay to sort and culture memory T cells. Four weeks after vaccination, we purified and cultured the CD44highCD62L+(central memory), CD44highCD62L-(effector memory), and CD44low/int (antigen-experienced) cell populations using FACS (Fig. 3e). We evaluated the frequency of antigen-specific CD4 vs. CD8 T cells that expanded from each sorted population and found that while CD44low/int cells failed to expand in response to antigen-pulsed feeder cells, both central and effector memory cell populations did expand (Fig. 3f). Regardless of the inclusion of adjuvant, there was a strong bias toward central memory cells being predominantly CD8 T cells while effector memory cells were predominantly CD4 T cells. No clear difference between the adjuvants was observed. Additionally, no clear impact on the skewing of the central vs. effector memory populations by adjuvants was observed in either CD4 or CD8 T cells.

Overall our studies found that the frequency of antigen-specific T cells appeared to correlate with efficacy, but the specific role of CD4 and CD8 T cells was unclear. To further understand the relevance of CD8 T cells, we examined vaccination in CD8-deficient mice.

3.4. CD8 T Cells are not Required for VLP-Mediated Protection, with or without Adjuvants

Protection against some pathogens, including HIV-1, malaria, and even influenza, is associated not only with antibody responses but with the frequency of Th1 profile, cytotoxic T cell responses (Watkins, 2008; Doll and Harty, 2014; Sridhar et al., 2013; Mendoza et al., 2013; Quinn et al., 2013a). CD8 T cell responses may also be critical for protection mediated by the adenovirus-based Ebola vaccine (Sullivan et al., 2011), although other filoviruses do not appear to require an antigen-specific CD8 T cell response (Rao et al., 2002; Marzi et al., 2013; Wong et al., 2012). To determine whether the presence of antigen-specific CD8 T cells is necessary for VLP-mediated durable protection, we vaccinated C57BL/6 mice and CD8-deficient mice with VLP, with or without polyICLC or CpG. Animals were challenged four weeks or twenty-two weeks after the vaccine boost. The lack of CD8 T cells had no effect on animal survival or on anti-GP IgG titers. All vaccinated animals survived short term challenge, with the exception of one CD8-deficient animal vaccinated with VLP alone (Fig. 4a). Additionally,
the durable challenge model, all (10/10) animals vaccinated with VLP and polyICLC and 90% (9/10) of animals vaccinated with VLP and CpG survived, as did 44% (4/9) of C57BL/6J animals vaccinated with VLP alone and 30% (3/10) of CD8-deficient animals vaccinated with VLP alone (Fig. 4b). Notably, it appears that CpG was more effective in this C57BL/6J strain than in the C57BL/6 mice used for other studies; nonetheless, the trends between the CD8-deficient animals and the control animals were comparable.

To determine whether a compensatory immune response was accounting for survival in the CD8-deficient animals, we examined antigen-specific antibody and CD4 T cell frequencies. Anti-GP IgG titers between CD8-deficient animals and C57BL/6J animals did not differ at
either time point (Fig. 4c). Additionally, there were minimal differences in the frequency of antigen-specific CD4 T cells between mouse strains (Fig. 4d). These data suggest that the survival observed in the CD8-deficient animals was not attributable to a compensatory increase in the CD4 T cell population or an enhanced antibody response due to potentially higher frequencies of helper CD4 T cells, and they imply that CD8 T cells are not required for VLP-mediated protection in the mouse model.

3.5. Adjuvants Associated with Durable Protection Enhance Tfh Cell Frequency

In our study, the protective vaccination regimen resulted in an increase in the frequency of antigen-specific, cytokine-positive CD4 T cells (Fig. 3b). Additionally, CD4-deficient animals did not survive upon vaccination with VLP alone \((p < 0.0001\) comparing survival of C57BL/6J vs CD4 \(-/-\)) and these animals were largely incapable of mounting an IgG response upon VLP vaccination, suggesting that antibody class switch recombination was T cell dependent (Supplemental Fig. 2).

In order to take a more global look at factors differentiating T cells after vaccination with VLP as compared to VLP and polyICLC, we vaccinated mice and isolated draining popliteal LN seven days after vaccination. Untouched T cells were isolated from the lymph nodes using bead depletion, and RNA was isolated, pooled, and subjected to analysis using the SABioscience T-cell and B-cell Activation Array (PAMM-053Z). Five transcripts were up-regulated more than two-fold in T cells from animals vaccinated with VLP and polyICLC as compared to VLP alone, with \(p\) values less than 0.05 (Fig. 5a). CXCR5 was one of these transcripts, and it was increased with a \(p\)-value of 0.0002. CXCR5 is

![Fig. 3. Effective adjuvants increase the frequency of antigen-specific T cells. (a) IFN-\(\gamma\) ELISPOT analysis of splenocytes from C57BL/6 mice vaccinated two times with indicated vaccine and adjuvant combination. Splenocytes were collected on day 4 after the second vaccination. Data is pooled from four separate experiments each containing 2–3 mice per group; median with IQR shown. (b) Frequency of IFN-\(\gamma\)+, IL2+, or TNF-\(\alpha\)+ cells after vaccination with VLP and polyICLC or VLP and CpG; median shown. Gating is on viable T cells and then CD4+CD44high T cells (c) or CD8+CD44high T cells (d). IFN-\(\gamma\) ELISPOT analysis of splenocytes from C57BL/6 mice vaccinated with indicated vaccine and adjuvant combination. Animals received the standard two vaccinations and then received a third vaccine boost 22 weeks after the second vaccination. Splenocytes were collected 4 days later. Data is pooled from two separate experiments each containing 3–4 mice per group; median with IQR shown. (e) Four weeks after the second vaccination, splenocytes were collected from animals and T cells were isolated using negative bead selection. T cells were then sorted to collect central memory, effector memory, and CD44int/low cell populations. Gating strategy is shown. (f) Sorted T cells were cultured with peptide-exposed, T cell-depleted, naïve splenocytes and the frequency of cytokine-positive CD4 and CD8 T cells in each sorted population was quantified after 5 days of culture. Gating is on CD4 or CD8 T cells and data shown are the frequency of cells expressing IFN-\(\gamma\), TNF-\(\alpha\), or IL2; median is shown. ** indicates \(0.01 < p < 0.05\).

upregulated in T follicular helper (Tfh) cells, which are critical for B cell class switch recombination.

To explore the impact of adjuvants on Tfh expansion, we vaccinated animals with adjuvant alone, VLP alone, or VLP in combination with each of the TLR agonist adjuvants. Seven days after vaccination, we euthanized mice and examined the frequency of Tfh cells (CXCR5^+PD1^+) in the draining popliteal LN. These cells expressed elevated ICOS and Bcl6 in comparison to CXCR5^- cells. Animals vaccinated with VLP and polyICLC had significantly higher frequencies of this cell population than those vaccinated with VLP alone (p = 0.0276), while animals vaccinated with VLP in combination with either MPLA or CpG exhibited intermediate frequencies of Tfh cells (Fig. 5b-c). These data point to a critical helper role for CD4 T cells in durable vaccination, which may account for the robust IgG responses observed in this model.

### 3.6. Adoptive Transfer of T Cells and Serum from Vaccinated Animals Confers Protection from Challenge

Having established that T cell responses were impacted by inclusion of protective adjuvants both in terms of cytokine-producing antigen-specific T cells and the frequency of Tfh, we examined whether adoptive transfer of T cells from vaccinated animals into naïve mice could confer protection from Ebola virus challenge. Cells and serum were collected from vaccinated animals 4 weeks after the second vaccination. T cell subsets were isolated by negative depletion so as to minimize inadvertent activation of T cells prior to transfer. Cells and serum, alone or in combination, were transferred into naïve mice (IP) 24 h prior to virus challenge.

As shown in Table 1, transfer of cells or serum from animals vaccinated with VLP alone had no impact on the survival of the recipient animals. Additionally, transfer of serum alone or splenocytes alone from animals vaccinated with VLP and polyICLC had no impact on the survival of the recipient animals. However, combining serum with cells from animals vaccinated with VLP and polyICLC was capable of rescuing survival, and transfer of as few as 5x10^6 splenocytes from vaccinated animals rescued survival in ~90% (9/10) of recipient animals (Table 1). To determine whether T cells were important for the cellular aspect of conferred protection, 5x10^6 T cells from VLP and polyICLC-vaccinated animals were transferred to naïve animals, with or without serum (Table 2). Not surprisingly, the T cells alone were unable to confer protection from challenge; however, 89% (8/9) of animals receiving serum and 5x10^6 T cells survived challenge. Again, transfer of cells and serum from animals vaccinated with VLP alone was not protective.

Data obtained from vaccination studies with CD8-deficient animals (Fig. 4) and the statistically significant increase in CD4 effector cells upon vaccination with VLP and polyICLC as compared to VLP and CpG (Fig. 3) led us to hypothesize that CD4 T cells would be critical for protection from challenge. To test this hypothesis, 4x10^6 CD4 or CD8 T cells

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**Fig. 4.** CD8 T cell deficiency does not impact short term or long term survival of vaccinated C57BL/6J mice. (a) Mice were treated IM twice with saline, VLP, VLP and polyICLC, or VLP and CpG. Four weeks after the second vaccination, mice were challenged. Closed symbols represent wild type C57BL/6J mice and open symbols indicate CD8-deficient mice. (b) Mice were vaccinated on the same schedule as in A, but challenge occurred 22 weeks after the second vaccination. (c) Two weeks after the second vaccination and 1 week prior to challenge, blood was collected from vaccinated animals and evaluated for anti-GP IgG antibody titers. (D) A subset of vaccinated animals was euthanized 4 days after the vaccine boost to evaluate CD4 + T cell responses. Median response of C57BL/6J mice and CD8-deficient mice is shown. N = 8–10 per treated group for A–C and D presents data pooled from two separate evaluations of 4–8 mice per group each. *** indicates 0.005 < p < 0.05.
from animals vaccinated with VLP and polyICLC were transferred into naïve animals. Ninety-two percent of animals receiving CD4 T cells and serum survived challenge while none of the animals receiving CD8 T cells and serum survived. To determine whether increasing the number of transferred CD8 T cells would impact the result, 12e6 CD8 T cells were transferred with serum; this combination protected 60% (3/5) of recipient animals (Table 2). These data suggest that the frequency of transferred cells is critical for mediating protection. Finally, to determine whether CD4 T cells from animals vaccinated with VLP and CpG could confer protection, serum and 4e6 CD4 T cells were transferred into naïve animals. Eighty percent of recipient animals survived challenge (Table 2).

### Table 1
Adoptive transfer of bulk splenocytes and serum from vaccinated animals to naïve animals.

| Group          | Transfer group (donor animals) | Number of cells | Cell type | Serum (μl) | Number of animals* | Percent survival | Challenge material | Challenge dose level |
|----------------|--------------------------------|-----------------|-----------|------------|--------------------|------------------|--------------------|---------------------|
| 1              | PolyICLC + VLP                  | 5e6             | Splenocytes | 200        | 10^4               | 90               | Ma-EBOV            | 1000 pfu            |
| 2              |                                 | 20e6            | Splenocytes | 200        | 10^4               | 83               | Ma-EBOV            | 1000 pfu            |
| 3              |                                 | 20e6            | Splenocytes | n/a        | 10^4               | 0                | Ma-EBOV            | 1000 pfu            |
| 4              |                                 | n/a             | n/a        | 200        | 5                  | 0                | Ma-EBOV            | 1000 pfu            |
| 5              |                                 | 5e6             | Splenocytes | 200        | 5                  | 0                | Ma-EBOV            | 1000 pfu            |
| 6              | VLP                             | 20e6            | Splenocytes | 200        | 5                  | 0                | Ma-EBOV            | 1000 pfu            |
| 7              |                                 | 20e6            | Splenocytes | 500        | 5                  | 0                | Ma-EBOV            | 1000 pfu            |
| 8              |                                 | n/a             | n/a        | n/a        | 60^*               | 0                | Ma-EBOV            | 1000 pfu            |

* Pooled from multiple iterations with n = 4 to 10 per iteration. Fisher’s exact test with Bonferroni correction, pairwise comparisons were used to evaluate Group 1 vs. 2 (p = 0.3819), 1 vs. 4 (p = 0.006), 1 vs. 5 (p = 0.006), 2 vs. 3 (p = 0.0005), and 2 vs. 6 (0.0044).
4. Discussion

Protein-based vaccines offer a safe and effective means to achieve protection from a variety of pathogens, but their poor immunogenicity makes inclusion of an adjuvant imperative. In addition to enhancing immunogenicity, adjuvants can provide dose sparing and impact the required vaccination schedule. In this study, we examined the impact of adjuvants on the durability of a model protein-based vaccine, the filovirus VLP.

Of the adjuvants tested in this system, polyICLC had the most beneficial impact on durable protection. However, vaccination with VLP and polyICLC impacted nearly every immune parameter tested, including NAb titers, Th1-sketched antibody titers, CD8 T cell responses, CD4 T cell responses, and Th1 cell responses. To delineate the relative importance of these immune parameters, we compared effects elicited by polyICLC to those elicited by the less effective antigen and adjuvant combinations.

In terms of antibody response, a Th1-skewed antibody response correlated with protection. Vaccination with VLP and polyICLC yielded significantly higher IgG2c titers than vaccination with any other vaccine and adjuvant combination. In contrast, vaccination with alhydrogel and VLP, which had no impact on survival, resulted in a Th2 skewed antibody response. To further examine the impact of antibody on survival, NAb titers were examined. NAb are associated with protection in several vaccine models (Roy et al., 2015; van Gils and Sanders, 2014; Kok et al., 2014; Plotkin, 2010), but data regarding the importance of NAb in EBOV protection are conflicting (Wong et al., 2012; Sullivan et al., 2009; Audet et al., 2014; Oswald et al., 2007; Grant-Klein et al., 2015; Lee et al., 2008; Bale et al., 2012; Martins et al., 2015a; Agnandji et al., 2015). Interestingly, NAb titers had no relationship to survival in this durable protection model. Both polyICLC and alhydrogel substantially impacted NAb titers and improved survival on a short term timescale; however, only polyICLC had a beneficial impact on durable protection. This examination of antibody feeds into a larger discussion on the role of neutralization and the definition of neutralizing antibody. The assay utilized in this study includes supplementation with complement, which complicates the definition of neutralization. Despite this fact, neutralization still did not correlate with survival while IgG2c titers did, suggesting an under-appreciated role for non-neutralizing antibody in controlling infection under a durable protection model.

Previous work has demonstrated that CD4 T cells may be important for Ebola-mediated protection (Wong et al., 2012; Rao et al., 2002). However, dissecting the role of CD4 T cells in protective immunity is challenging due to their impact on the development of high affinity antibody responses and effective CD8 T cell responses (Sant and McMichael, 2012). CD4 T cells also have an effector function and are critical for immune cell recruitment during infection (Soghoian and Streeck, 2010; McKinstry et al., 2012; Brown et al., 2012; Johnson et al., 2015). Recently, an appreciation for the importance of CD4 T cells in viral infection has grown, particularly in the context of influenza vaccination (Zeis and Farber, 2015). Antigen-specific CD4 T cells were associated with lower viremia during human influenza infection (Wilkinson et al., 2012), and, in nonhuman primates, CD4 T cell populations were important in Hepatitis A clearance (Zhou et al., 2012). Because of the complex role of CD4 T cells in the immune response, the impact of adjuvants on CD4 T cell responses has also been extensively examined (Baumgartner and Malherbe, 2010; McAleer and Vella, 2010). Both TLR agonist adjuvants and aluminum-containing adjuvants have been associated with CD4 T cell efficacy (Monaci et al., 2015; Sokolovska et al., 2007).

Three compelling sets of data from our studies suggest that, in combination with a Th1-skewed antibody response, CD4 T cells correlate with VLP-mediated protection under a durable protection murine model. First, the frequency of IFN-γ-producing T cells was significantly higher in the protective adjuvant vaccination (polyICLC + VLP) than the partially protective (CpG + VLP) or unadjuvanted vaccination, and these cells were predominantly CD4 T cells. Second, transcriptomic analysis of T cells from animals vaccinated with the protective vaccine revealed up-regulation of CXCR5 as compared to vaccination with VLP alone. This observation led us to examine the frequency of Th1 cells in the draining lymph node, where we observed that polyICLC significantly enhanced the frequency of Th1 cells over the suboptimal vaccinations. Third, adoptive transfer of CD4 T cells from animals vaccinated with VLP and polyICLC, in combination with serum, was protective at cell frequencies as low as 4e6 whereas three times as many CD8 T cells were required for comparable protection. In combination, these data suggest that it is not simply the impact of CD4 T cells on B cell maturation or CD8 T cell development that makes CD4 T cells critical for vaccine-mediated protection, but that the actual presence of antigen-specific CD4 T cells is relevant for protection from challenge.

The data presented here inform broadly on how the tested adjuvants impact the immune response to a protein antigen. Moreover, by testing these adjuvants in a challenge model, the data inform on the specific requirements for long term protection from ma-EBOV challenge. Describing the immune profile of these canonical TLR agonists may help direct adjuvant discovery work targeted at achieving long-lasting, vaccine-mediated protection.

Contributions

KM developed study design, implemented assays, analyzed data, and wrote manuscript. CC implemented some assays and contributed to writing. SS, SK, JS, JB, and SvT conducted experiments, and SS and SK did data analysis. SN did statistical analysis. SB contributed to project development.

Conflicts of Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jibiom.2015.11.041.

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