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Induction of Humoral and CD8⁺ T Cell Responses Are Required for Protection against Lethal Ebola Virus Infection¹

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Ebola virus (EBOV)-like particles (eVLP), composed of the EBOV glycoprotein and matrix viral protein (VP)40 with a lipid membrane, are a highly efficacious method of immunization against EBOV infection. The exact requirements for immunity against EBOV infection are poorly defined at this time. The goal of this work was to determine the requirements for EBOV immunity following eVLP vaccination. Vaccination of BALB/c or C57BL/6 mice with eVLPs in conjunction with QS-21 adjuvant resulted in mixed IgG subclass responses, a Th1-like memory cytokine response, and protection from lethal EBOV challenge. Further, this vaccination schedule led to the generation of both CD4⁺ and CD8⁺ IFN- γ ⁺ T cells recognizing specific peptides within glycoprotein and VP40. The transfer of both serum and splenocytes, but not serum or splenocytes alone, from eVLP-vaccinated mice conferred protection against lethal EBOV infection in these studies. B cells were required for eVLP-mediated immunity to EBOV because B cell-deficient mice vaccinated with eVLPs were not protected from lethal EBOV challenge. We also found that CD8⁺, but not CD4⁺, T cells are absolutely required for eVLP-mediated protection against EBOV infection. Further, eVLP-induced protective mechanisms were perforin-independent, but IFN- γ -dependent. Taken together, both EBOV-specific humoral and cytotoxic CD8⁺ T cell responses are critical to mediate protection against filoviruses following eVLP vaccination. *The Journal of Immunology*, 2005, 175: 1184–1191.

Many virus-like particle (VLP)³ vaccines are being developed to protect against viral infections including those caused by human papillomavirus, parvovirus, rotavirus, Norwalk virus, and hepatitis B virus (1–3). VLPs depend on the natural ability of the viral protein (VP) to self-associate and form particles that are morphologically similar to the live virus from which the proteins are derived (4). Administration of VLPs can trigger the induction of neutralizing Abs and virus-specific CD8⁺ CTL (1–3). Therefore, VLPs are capable of mobilizing different arms of the adaptive immune system, are safe and effective in clinical settings (5, 6), and raise no preexisting or vector immunity concerns associated with other vaccine strategies.

The members of the family Filoviridae, Ebola virus (EBOV) and Marburg virus (MARV), are enveloped, nonsegmented, negative-stranded RNA viruses (7). Natural outbreaks are sporadic and cause severe hemorrhagic fevers (8). The reservoir of EBOV and MARV is unknown, although cases occur most often due to contact with blood or tissues from infected monkeys or humans (9, 10). Recent outbreaks in humans appear to stem from an index case coming into contact with a single infected monkey (9, 11).

Filoviruses are among a group of pathogens that present a clear biological warfare threat, due to their high mortality rate (50–90%), stability, ease of mass production, and ability to infect via aerosol or droplet transmission (7). Unfortunately, there is a general lack of understanding regarding the requirements for immunity against filoviruses, especially in humans and nonhuman primates (12). Hence, at this time, there are no licensed vaccines, prophylactics, or treatments for filovirus infections in humans. Recently, several vaccine regimens have elicited protective immunity against filoviruses in nonhuman primates, although the correlates of immunity for these vaccine approaches are not fully characterized (13, 14). Because human efficacy studies are not feasible for EBOV and MARV vaccine candidates, surrogate markers of immunity will have to be identified in animal studies.

In specific instances, EBOV-specific Abs can be sufficient to protect against lethal filovirus disease. In rodents, Abs with the appropriate specificity, isotype, and avidity are sufficient to protect against filovirus infection (12, 15, 16). Transfer of GP-specific Abs immediately before or as late as 2 days after challenge confers protection against EBOV challenge in rodents; transfer of immune serum specific for VPs other than GP were less efficacious (16, 17). Thus far, treatment with EBOV-immune sera delays, but does not provide protection against lethal EBOV infection in monkeys (18–20). Humans that survive filovirus infections have an early proinflammatory response and a persistent rise in anti-EBOV IgG, whereas humans that succumb to infection do not develop detectable virus-specific Ab (21, 22). In some instances, high levels of EBOV-specific Ab correlate with protection and in others it does not; therefore, the exact contribution of Ab in protection against EBOV infection is not clear.

The role of T cell responses in protection against filovirus infection is also not well defined, but it is generally accepted that cellular immune responses are integral for achieving protection against filovirus infection (12). Both CD4⁺ and CD8⁺ T cells are proposed to be critical for protection against EBOV (12, 20, 23).

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³ Abbreviations used in this paper: VLP, virus-like particle; EBOV, Ebola virus; eVLP, Ebola virus-like particle; NP, nucleoprotein; MARV, Marburg virus; VP, viral protein; β_2m , β_2 -microglobulin.

For EBOV, several vaccine strategies including liposomes encapsulating inactivated EBOV, DNA alone, DNA prime/adenovirus boost, and alphavirus replicon vaccines induce CTL responses against EBOV-specific epitopes of GP and/or nucleoprotein (NP) in mice (20, 23–26). The importance of CTL responses was further demonstrated by successful adoptive transfer of NP-specific CTLs (23). Humans who succumb to EBOV infection seem to have no detectable CTL responses, whereas survivors develop activated CTLs shortly following infection (21).

Ebola VLP (eVLP) and Marburg VLP self-assemble and bud from cellular lipid rafts following expression of GP and VP40 in mammalian cells (27–32). Mice and guinea pigs vaccinated with eVLPs are completely protected from lethal EBOV challenge (28, 29, 33); therefore, eVLPs represent a promising vaccine candidate for prevention of lethal EBOV infections. In this study, the mouse model of EBOV infection was used to examine the components of the immune responses that are required for eVLP-mediated protective immunity, which included humoral, cellular, and cytokine responses.

Materials and Methods

Virus

EBOV Zaire 1995 virus preparations were purified and inactivated by irradiation with 1×10^7 rad. All EBOV-infected cells and mice were handled under maximum containment in a biosafety level (BSL)-4 laboratory at the U.S. Army Medical Research Institute of Infectious Diseases (Frederick, MD). All samples removed from the BSL-4 laboratory were gamma irradiated with $2\text{--}10 \times 10^6$ rad before analysis in BSL-2 or BSL-3 laboratory.

Mice

$\beta\delta$ ($\alpha\beta$ and $\gamma\delta$ T cell) TCR-deficient or IFN- γ -deficient mice were obtained from The Jackson Laboratory. Jh B cell-, CD4⁺ T cell-, β_2 -microglobulin (β_2m)-, or perforin-deficient mice were purchased from Taconic Farms. Wild-type C57BL/6 or BALB/c mice were obtained from National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). All mice were 8–10 wk old at the start of the experiment, both female and male mice were used, and mice were randomly divided into treatment groups. Mice were housed in microisolator cages and provided autoclaved water and chow ad libitum. Mice were challenged by i.p. injection with 1000 PFU ($\sim 30,000$ LD₅₀) of mouse-adapted EBOV diluted in PBS (34). After challenge, mice were observed at least twice daily for illness.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the 1996 Guide for the Care and Use of Laboratory Animals (National Research Council). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Ebola VLPs

eVLPs were prepared essentially as previously described, with minor modifications (27–29, 35). Briefly, 293T cells were cotransfected using Lipofectamine 2000 (Invitrogen Life Technologies) with pWRG vectors encoding EBOV VP40 and GP, kindly provided by C. Schmaljohn (U.S. Army Medical Research Institute of Infectious Diseases). To purify the eVLPs, the clarified cell supernatants were pelleted, separated on a 20–60% continuous sucrose gradient, concentrated by a second centrifugation, and resuspended in endotoxin-free PBS. The gradient fractions containing the eVLPs were determined using Western blotting and electron microscopy. The total protein concentration of each eVLP preparation was determined in the presence of Nonidet P-40 detergent using a detergent-compatible protein assay (Bio-Rad). The eVLP preparations used in this study contained <0.03 endotoxin U/mg, as determined using the *Limulus* ameocyte lysate test (BioWhittaker).

Vaccinations

Mice were vaccinated i.m. with $10 \mu\text{g}$ of eVLPs mixed with $10 \mu\text{g}$ of QS-21 adjuvant (kindly provided by Antigenics) diluted in endotoxin-free PBS twice at 3-wk intervals. Control mice were vaccinated on the same

schedule with $10 \mu\text{g}$ of QS-21 adjuvant in PBS or PBS alone. Mice were challenged with EBOV 6 wk after the second vaccination.

Ab titers

Blood samples were obtained from the retro-orbital sinus under anesthesia, lateral tail vein, or by cardiac puncture under anesthesia, and serum was collected and stored at -70°C . Levels of EBOV-specific Abs were determined, as previously described (36). Briefly, the wells were coated with sucrose-purified inactivated EBOV. Serial 3-fold dilutions of individual or a pool of serum were tested, detected using an HRP-conjugated Ab to measure total (IgA, IgG, IgM; Sigma-Aldrich) or the individual isotype (IgA or IgM) or IgG subclass Ab levels (Southern Biotechnology Associates), and developed using tetramethylbenzidine substrate. Ab titers were defined as the reciprocal of the highest dilution showing a net OD ≥ 0.2 .

Proliferation assay

Splenocytes of individual mice vaccinated with QS-21 or eVLP mixed with QS-21 were harvested 2 wk following their second vaccination and were placed in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 1 mM HEPES, and 0.1 mM nonessential amino acids (complete medium). Contaminating RBC were lysed with ACK lysing buffer (Quality Biologicals) and the erythrocyte-free splenocytes were washed twice with RPMI 1640 complete medium. Cells (200,000) were plated in each well of a 96-well culture plates in RPMI 1640 complete medium alone or with $10 \mu\text{g}/\text{ml}$ eVLP or inactivated EBOV, as indicated. After 5 days in culture, $100 \mu\text{l}$ of the culture supernatants were removed for cytokine analysis and $1 \mu\text{Ci}$ of [³H]thymidine in $100 \mu\text{l}$ of medium was added to each well. After 18 h, the plates were subjected to a freeze-thaw cycle and ³H incorporation was determined.

Cytokine detection

To determine the levels of cytokines in the supernatant of restimulated cells, cytometric bead assays (BD Biosciences) were used to detect IL-2, IL-4, IL-5, IFN- γ , or TNF- α , per the manufacturer's directions. All flow cytometry data were acquired with a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Immunocytometry Systems).

To determine epitopes recognized by CD4⁺ and CD8⁺ T cells from eVLP-vaccinated mice, EBOV-specific responses were analyzed by culturing splenocytes with 1–5 μg of overlapping (by 10 aa) 15-residue peptides representing the GP or VP40 of EBOV (Mimotopes) or PMA (25 ng/ml) and ionomycin (1.25 $\mu\text{g}/\text{ml}$) in $100 \mu\text{l}$ of RPMI 1640 medium containing $10 \mu\text{g}/\text{ml}$ brefeldin A (Epicentre Technologies) at 37°C for 5 h. In some cases, 8–10 residue peptides were used to narrow down epitope responses following positive initial results with 15-residue peptides. After culture, the cells were blocked with mAbs to FcRIII/II and stained with anti-CD44 FITC, anti-CD8 CyChrome, or anti-CD4 CyChrome (BD Pharmingen) in staining wash buffer (PBS, 2% FBS, 0.01% sodium azide) with brefeldin A. The cells were fixed in 1% formaldehyde, permeabilized with staining wash buffer plus 0.5% saponin (Sigma-Aldrich), and stained with anti-IFN- γ PE (BD Pharmingen).

Adoptive transfers

For transfer experiments, BALB/c mice were vaccinated twice (3-wk interval) with $10 \mu\text{g}$ of eVLP and $10 \mu\text{g}$ of QS-21 or with $10 \mu\text{g}$ of QS-21 alone. Serum or splenocytes were harvested from half the mice 6 wk after the last vaccination. The samples from each group were pooled. A portion of the splenocytes were set aside and transferred as unfractionated. The remaining splenocytes were divided into two sets and enriched either for B or T lymphocytes. The B cell-enriched population was generated by first incubating the splenocytes with rat anti-mouse CD3, CD4, CD8 α , CD11b, DX5, and Gr-1 (BD Biosciences). Similarly, the T cell population was enriched using rat anti-mouse CD11c, CD19, DX5, and Gr-1 (BD Biosciences). The unwanted cells were removed from each preparation by addition of sheep anti-rat IgG magnetic beads (DynaL Biotech). The B cell population contained $>97\%$ B220⁺ cells and the T cell population contained $>95\%$ CD5⁺ cells following enrichment. Three days before challenge, naive BALB/c mice received 0.5 ml of serum, 2×10^7 unfractionated splenocytes, both serum and splenocytes, or 1×10^7 B cell- or T cell-enriched preparations from mock- or VLP-vaccinated mice via tail vein injection. The recipient mice or VLP-vaccinated mice were challenged with 1000 PFU of mouse-adapted EBOV 3 days after the transfer, or 6 wk after the last vaccination, respectively.

Data analysis

Differences in proliferation or cytokine secretion were determined using a paired Student *t* test. Correlations between protection and Ab levels were

determined using Pearson's test. Statistical significance was set at $p \leq 0.05$.

Results

Vaccination with eVLPs in QS-21 adjuvant induces broad Ab responses and protection

We previously showed that 3 doses of 10 μg of eVLPs in the absence of adjuvant administered to mice at 3-wk intervals induced protection from EBOV challenge (28). Mice were vaccinated twice with 10 μg of eVLPs in the presence of the saponin derivative QS-21, to shorten the vaccination regimen by 3 wk. Vaccination with eVLPs and QS-21 induced a mixed class and subclass EBOV-specific Ab response in the serum of eVLP-vaccinated BALB/c and C57BL/6 mice, with the IgG1 being produced in the highest amounts, followed by IgG2a and IgG2b, and then IgG3 and IgM (Fig. 1A). No EBOV-specific IgA was detected in the serum of eVLP-vaccinated mice (Fig. 1A). Mice vaccinated

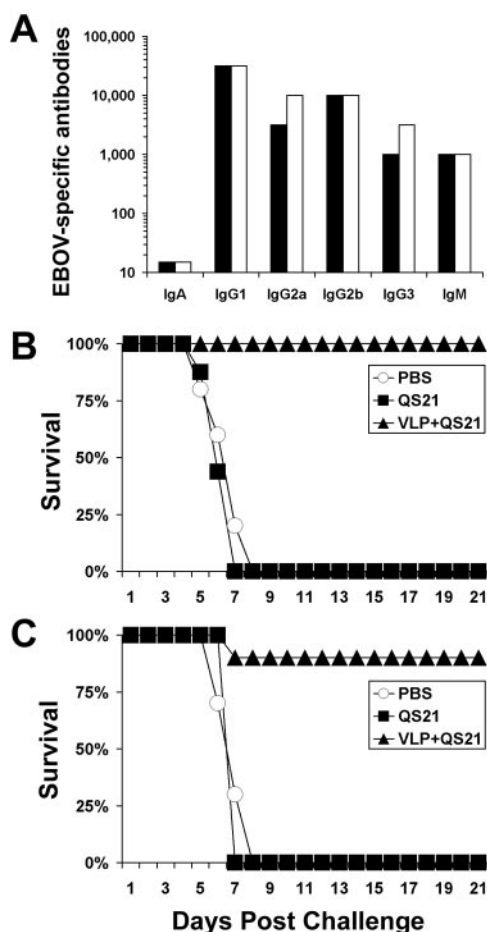


FIGURE 1. Serum Ab responses and protection following vaccination of mice with eVLPs. Wild-type BALB/c (□) or C57BL/6 (■) mice were vaccinated with 10 μg of eVLPs and 10 μg of the saponin derivative QS-21, 10 μg of QS-21, or PBS alone twice at 21-day intervals. A, Serum was collected 6 wk following the last eVLP vaccination. The levels of total, IgA, IgM, or IgG subclass anti-EBOV-specific Abs in pools of sera from five individual mice were measured by ELISA. BALB/c or C57BL/6 mice vaccinated with QS-21 alone or PBS alone did not develop detectable EBOV-specific Abs (data not shown). B and C, The mock-vaccinated (QS-21 or PBS) or VLP-vaccinated BALB/c (B) and C57BL/6 (C) mice were challenged 6 wk after the second vaccination with 1000 PFU of mouse-adapted EBOV. Results are plotted as percentage of survival for each vaccination group ($n = 10$ per group). These data are representative of two experiments of similar design and outcome.

with PBS or QS-21 alone did not develop detectable EBOV-specific Abs (data not shown). This new vaccine regimen provided 100% protection in BALB/c (Fig. 1B) and 90% protection in C57BL/6 mice following a challenge dose of 1000 PFU of EBOV (Fig. 1C). Mice vaccinated with QS-21 alone or PBS did not survive lethal EBOV challenge (Fig. 1, B and C).

eVLP vaccination induces recall proliferative and cytokine responses

T cells are activated transiently following eVLP vaccination in mice (28). Therefore, we first analyzed the proliferative and cytokine recall responses of T cells following eVLP vaccination. To this end, splenocytes were isolated from C57BL/6 mice vaccinated with eVLP and QS-21 adjuvant or QS-21 alone and were stimulated in vitro with medium alone, IL-2, eVLP, or inactivated EBOV for 5 days. A significant increase in the proliferative response was observed when splenocytes from eVLP-vaccinated mice were cultured with eVLPs, as compared with splenocytes cultured with medium alone (Fig. 2A). In sharp contrast, splenocytes from mice vaccinated with eVLPs did not respond in culture to inactivated EBOV (Fig. 2A). As expected, splenocytes from mice vaccinated with either eVLPs in QS-21 or with QS-21 alone proliferated in the presence of exogenously added IL-2, whereas splenocytes from QS-21-vaccinated mice did not respond to eVLPs or inactivated EBOV (Fig. 2A). To determine the cytokine profiles of eVLP-vaccinated mice, the supernatants from the cultured splenocytes were tested for secretion of IFN- γ or IL-4. Upon stimulation with IL-2, splenocytes from mice vaccinated with either eVLP and QS-21 or QS-21 alone secreted IFN- γ , but not IL-4 (Fig. 2, B and C). Upon restimulation with eVLPs, splenocytes from mice vaccinated with eVLP and QS-21 secreted significantly more IFN- γ than splenocytes from QS-21-vaccinated mice, but cells from neither group secreted IL-4 (Fig. 2 B and C). Cultures with inactivated EBOV did not induce significant secretion of cytokines from splenocytes of either eVLP- or mock-vaccinated mice (Fig. 2, B and C). Our next goal was to further characterize the IFN- γ responses of CD4⁺ and CD8⁺ lymphocytes to GP and VP40.

VLP vaccination induces Ebola GP- and VP40-specific T lymphocyte responses

Our previous findings demonstrated that both CD4⁺ and CD8⁺ lymphocytes are stimulated after VLP vaccination (28). To further evaluate the CD4⁺ and CD8⁺ EBOV-specific responses following VLP vaccination, splenocytes from vaccinated C57BL/6 and BALB/c mice were restimulated with pools of overlapping peptides from either EBOV GP or VP40. Specific CD8⁺ T cell responses were detected in eVLP-vaccinated BALB/c and C57BL/6 mice using IFN- γ production as a marker (Table I). In BALB/c mice, a GP-specific CD8⁺ response was generated to the amino acid sequence LYDRLASTV (GP₁₆₁₋₁₆₉), a sequence that was previously described by Rao et al. (20, 37). A response to GP₁₆₁₋₁₆₉ in a representative set of mice is shown in Fig. 3, as compared with the nonspecific response to a Lassa NP peptide (Fig. 3 and Table I). As shown in Table I, two CD8⁺ T cell responses were detected to the VP40 sequences YFTFDLTALK (VP40₁₇₁) and TESPEKIQAI (VP40₂₃₂). In addition, the VP40 peptide sequence from amino acid position 102 to 116 (PEYMEAIYPVRSNST) induced IFN- γ production in CD4⁺ T lymphocytes. Analysis of VLP-vaccinated C57BL/6 mice revealed a single peptide sequence within VP40₁₅₀ LRIGNQAFLEFVL that was capable of inducing IFN- γ production in CD8⁺ cells. A single amino acid sequence within GP stimulated IFN- γ production in both CD8⁺ and CD4⁺ T cells from C57BL/6 mice (Table I).

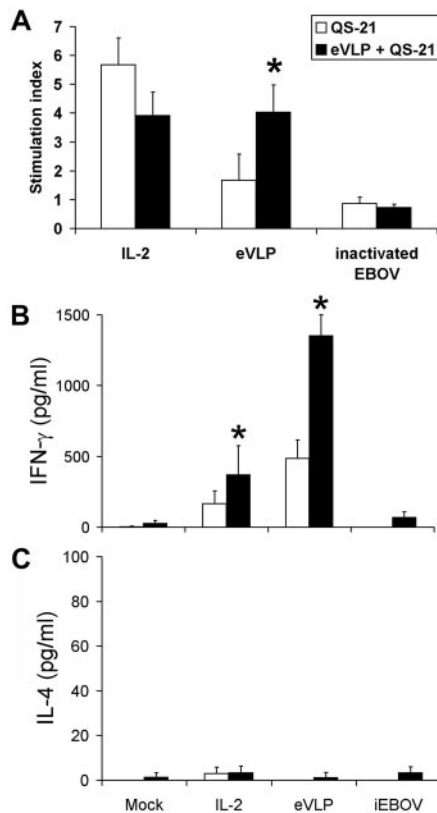


FIGURE 2. Ebola VLPs induce Th1-like memory immune responses. *A*, Splenocytes from C57BL/6 mice ($n = 5$) vaccinated with eVLPs and QS-21 (■) or QS-21 alone (□) were stimulated in vitro with medium alone, IL-2, eVLP, or inactivated EBOV for 5 days. During the last 18 h of culture, [3 H]thymidine was added to each well and the amount of 3 H incorporation was assessed. These data are presented as the stimulation index, which was determined by dividing the 3 H incorporation in wells stimulated with Ag by the 3 H incorporation of wells cultured with medium alone. The error bars represent the SD of the mean of the stimulation index. *, Significant differences in the proliferation ($p < 0.05$) between the eVLP-vaccinated mice and adjuvant alone-vaccinated mice, as determined by paired t test. *B* and *C*, Mean levels of IFN- γ (*B*) or IL-4 (*C*) secretion in the culture supernatants of the splenocytes were determined using cytometric bead assay. The error bars represent one SD from the mean. *, Significant differences in cytokine secretion ($p < 0.05$) between the eVLP-vaccinated mice and mock-vaccinated mice are shown and were determined using a paired t test.

Surviving EBOV infection is tightly linked to both humoral and cellular immune responses

Because eVLPs activate both B and T lymphocyte responses in mice (28) (Figs. 1 and 2), we wanted to determine which immune components were required for eVLP-mediated protection. To this end, groups of 20 mice were injected twice with eVLPs and QS-21 or with QS-21 alone, and after 6 wk were used as donor mice. Serum or unfractionated splenocytes were harvested from eVLP-vaccinated mice and then transferred to naive recipient mice before lethal EBOV challenge. Neither the transfer of pools of sera nor of unfractionated splenocytes conferred protection to naive mice against EBOV (Table II). Similarly, groups of recipient mice receiving B or T lymphocyte-enriched cell populations from the eVLP-vaccinated mice were not protected against EBOV challenge (Table II). In contrast to administration of any of the immune components alone, transfer of both immune sera and splenocytes protected naive, recipient mice from EBOV infection (Table II). Also, as expected, mice that were administered eVLPs alongside

the donor mice were completely protected from EBOV challenge (Table II).

To directly determine which immune components could mediate protection from EBOV challenge following eVLP vaccination, we vaccinated genetically modified knockout mice. Jh mice, devoid of mature B cells in the periphery, were vaccinated twice with eVLPs and QS-21 adjuvant. Unlike eVLP-vaccinated BALB/c mice, B cell-deficient mice did not develop EBOV-specific Abs (Fig. 4A). B cells were required for eVLP-mediated immunity to EBOV because B cell-deficient Jh mice vaccinated with eVLPs were not protected from lethal EBOV challenge (Fig. 4B). In contrast, genetic-matched BALB/c mice were completely protected by eVLP vaccination (Fig. 4B). Neither B cell-deficient nor BALB/c mice vaccinated with QS-21 adjuvant alone survived lethal EBOV challenge (Fig. 4B).

$\beta\delta$ TCR-deficient mice (devoid of both $\alpha\beta$ and $\gamma\delta$ T cells), β_2m -deficient mice (lacking CD8 $^+$ T cells and designated CD8 $^+$ T cell-deficient), and CD4 $^+$ T cell-deficient mice all developed EBOV-specific Abs following eVLP vaccination, albeit at different levels (Fig. 4C). However, the eVLP-vaccinated CD4 $^+$, CD8 $^+$, or $\beta\delta$ T cell-deficient mice developed Ab levels that were generally lower than wild-type C57BL/6 mice (Fig. 4C). Despite development of EBOV-specific Ab responses, neither the $\beta\delta$ TCR-deficient mice, nor CD8 $^+$ T cell-deficient mice were protected by eVLP vaccination (Fig. 4D). In contrast, one-half of CD4 $^+$ T cell-deficient mice vaccinated with eVLPs survived EBOV challenge, but we observed no correlation between their EBOV-specific Ab titers and survival (data not shown). Both the CD4 $^+$ and CD8 $^+$ T cell-deficient mice that succumbed to EBOV disease had delayed time-to-death compared with naive mice (Fig. 4D), and we speculate that their high levels of circulating Abs held off early virus replication, but were not sufficient to mediate clearance of the infection (Fig. 4C).

Because CD8 $^+$ T cells were absolutely required for eVLP-mediated protection from EBOV infection, we exploited knockout mice to further examine the mechanisms of immunity. The antiviral activity of CD8 $^+$ T cells is mediated via direct killing, induction of apoptosis of the infected target cell, or secretion of cytokines (38). Therefore, perforin- or IFN- γ -deficient or wild-type C57BL/6 mice were vaccinated with eVLPs. All three genotypes of mice developed equivalent levels of total circulating EBOV-specific Abs following eVLP vaccination (Fig. 4E). However, following challenge with EBOV, only 2 of 15 eVLP-vaccinated IFN- γ -deficient mice survived (Fig. 4F). In contrast, wild-type C57BL/6 or perforin-deficient mice vaccinated with eVLPs exhibited nearly complete protection from lethal EBOV infection (Fig. 4F).

To determine whether the generation of a specific type of Ab correlated with protection, we evaluated EBOV-specific Abs in the eVLP-vaccinated knockout mice. eVLP-vaccinated C57BL/6 and BALB/c mice generated high titers of EBOV-specific IgG subclass Abs, as well as IgM (Fig. 5). As expected, B cell-deficient Jh mice

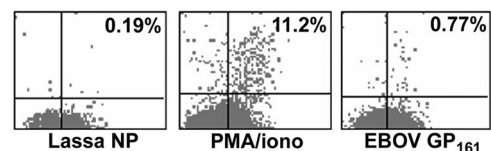


FIGURE 3. T cell responses in eVLP-vaccinated mice. Pooled splenocytes from three eVLP-vaccinated mice were restimulated in vitro with an EBOV-specific GP₁₆₁₋₁₆₉, a Lassa NP peptide, or PMA/ionomycin. The cells were stained after 4 h in culture with anti-CD44 FITC and anti-CD8 CyChrome. The cells were then permeabilized and stained with anti-IFN- γ PE. The percentage of CD8 $^+$, CD44 $^+$, and IFN- γ $^+$ cells is indicated in the top right quadrant of each plot.

Table I. *Ebola virus-specific T lymphocyte responses to GP and VP40 following vaccination with eVLPs^a*

Mouse Strain	EBOV Protein	Amino Acid Position	Epitope Sequence	Responding T Cells	Intracellular Cytokine Response (Sample/Control) ^b
BALB/c	GP	161–169	LYDRLASTV	CD8	0.77/0.19
		171–180	YFTFDLTALK	CD8	0.80/0.19
		232–241	TESPEKIQAT	CD8	0.73/0.19
C57BL/6	GP	102–116	PEYMEAIYPVRSNST	CD4	0.18/0.06
		531–545	WIPYFGPAAEGIYTE	CD8	0.11/0.04
		531–545	WIPYFGPAAEGIYTE	CD4	0.14/0.05
	VP40	150–164	LRIGNQAFLEQFVLP	CD8	0.13/0.04

^a BALB/c or C57BL/6 mice were vaccinated twice (3-wk interval). Splenocytes from eVLP-vaccinated animals were collected 7–14 days after the second vaccination and used ex vivo for identification of peptides that induced IFN- γ -expressing CD44⁺ and CD4⁺ or CD8⁺ T cells. In some cases, the epitope sequences recognized in the eVLP-vaccinated mice were narrowed down by the use of 9–10 residue peptides.

^b Percentage of IFN- γ -producing cells that were greater than 2-fold higher than background (no peptide or irrelevant peptide) were considered positive. These data are representative of at least two experiments performed with each peptide.

did not develop EBOV-specific Abs and T cell-deficient mice developed only detectable circulating IgM, but not any IgG subclass EBOV-specific Abs (Fig. 5). The CD4⁺ and CD8⁺ T cell-deficient mice developed lower levels of all the classes and subclasses tested, compared with wild-type C57BL/6 mice vaccinated with eVLPs (Fig. 5). eVLP-vaccinated IFN- γ - and perforin-deficient mice developed similar levels of EBOV-specific Abs, as compared with eVLP-vaccinated C57BL/6 mice, with the exception that IFN- γ -deficient mice did not develop EBOV-specific IgG2a (Fig. 5). None of the eVLP-vaccinated wild-type or knockout mice developed EBOV-specific IgA (Fig. 5). Mice vaccinated with QS-21 alone did not develop detectable EBOV-specific Abs of any type (data not shown). Together with previous data, our work suggests that the amount, specificity, and avidity of Abs toward EBOV will play a role in vaccine-induced protection from infection.

Discussion

VLPs are subunit vaccines with the distinct advantage over other candidate filovirus vaccines because they mimic the morphology and structure of the authentic virus from which they are derived. The use of adjuvant in the current studies allowed successful shortening of our vaccine regimen compared with administration of eVLP without adjuvant. Administration of 2 doses of eVLPs with QS-21 adjuvant resulting in >10-fold increases in circulating EBOV-specific Abs was compared with an equivalent vaccine regimen using eVLPs without adjuvant (data not shown) and equiv-

alent levels of high protection from lethal EBOV challenge (Fig. 1) (28). Ebola VLPs effectively induce both B cell and T cell responses, including EBOV-specific IgG, neutralizing Abs, and virus-specific CD4⁺ and CD8⁺ T cell responses. The exact contribution of these EBOV-specific responses to eVLP-mediated protection was not known. Using transfer experiments and knockout mice, we sought to define the immune components that are required for EBOV protection following eVLP vaccination. In the amount given in this study, neither serum nor unfractionated splenocytes from eVLP-vaccinated mice alone were able to protect naive recipient mice from EBOV challenge. However, administration of both serum and splenocytes from eVLP-vaccinated mice to naive mice provided protection from lethal EBOV infection. Further, eVLP vaccination did not provide any protection for B cell- or CD8⁺ T cell-deficient mice from EBOV challenge. Taken together, these data indicate that both humoral and CD8⁺ T cells are responsible for eVLP-mediated protection.

Because transfer of unfractionated splenocytes from immune mice did not confer protection to naive mice, it is highly suggestive that, in combination with CD8⁺ T cells, Abs and not the B cells themselves are mediating protection. Our finding that EBOV-specific immune sera alone is insufficient for protection is supported by previous studies in which high levels of neither total nor neutralizing Abs correlated with protection (13, 15, 16, 19, 28, 39). In contrast, administration of monoclonal EBOV-specific Abs protected mice and guinea pigs from EBOV challenge (16, 17). Therefore, a sufficient amount of Abs of a particular specificity, isotype, and avidity may prolong survival and give the host time to mount an effective immune response to the virus. The anti-GP mAbs that were efficient in protecting mice from EBOV challenge were predominantly IgG2a (16). In fact, despite the polarized IFN- γ memory response (Fig. 2C), all the IgG subtypes appear to be induced in fairly equivalent amounts (Fig. 1A). This is surprising due to the lack of IgG1 in IL-4-deficient mice (40); however, it is likely that a mixture of cytokines are produced during the vaccination process and this drives the mixed Ab profile in VLP-vaccinated mice. It is possible that larger amounts of eVLP-specific IgG2a might be able to confer protection from EBOV challenge following passive transfer. As expected, IFN- γ -deficient mice vaccinated with eVLPs did not develop IgG2a and showed no protection from EBOV challenge. However, whether the lack of IgG2a in the eVLP-vaccinated IFN- γ -deficient mice is directly linked to their lack of protection, or whether the IFN- γ is critical for T cell responses following challenge remains to be studied.

In contrast to the previous studies with mAbs the transfer of sera from eVLP-vaccinated mice did not induce protection; this may be

Table II. *Transfer of individual immune components from VLP-vaccinated BALB/c mice is not sufficient for protection from EBOV*

Treatment	Vaccine ^a	Survivors/Total ^b	Mean Time to Death ^b
Direct vaccination ^c	VLP + QS-21	20/20	N/A
Sera + splenocytes ^{d,e}	VLP + QS-21	9/10	N/A
Sera ^d	VLP + QS-21	0/20	6.1 \pm 0.9
Splenocytes ^e	VLP + QS-21	0/15	6.7 \pm 1.0
T cell-enriched ^e	VLP + QS-21	0/9	6.3 \pm 1.3
B cell-enriched ^e	VLP + QS-21	0/9	6.0 \pm 1.2
Naive ^{b,f}	QS-21	0/35	6.5 \pm 0.7

^a Donor BALB/c mice were vaccinated twice at 3-wk intervals with 10 μ g of eVLP and 10 μ g of QS-21 or 10 μ g of QS-21 alone and then rested for 6 wk.

^b Recipient BALB/c mice were challenged 3 days after transfer or 6 wk after the last vaccination with ~1000 PFU of EBOV, and survival was scored for 28 days.

^c A subset of the donor mice were used to verify that the vaccination regimen was sufficient protection in this particular experiment.

^d Sera (500 μ l) from donor mice were i.v. transferred.

^e Unfractionated splenocytes (20 \times 10⁶) or B cell- or T cell-enriched splenocytes (10⁷) were i.v. transferred.

^f Mice directly vaccinated with QS-21 (n = 10) or recipient mice receiving sera or unfractionated splenocytes from QS-21-vaccinated mice (n = 5 each) were used.

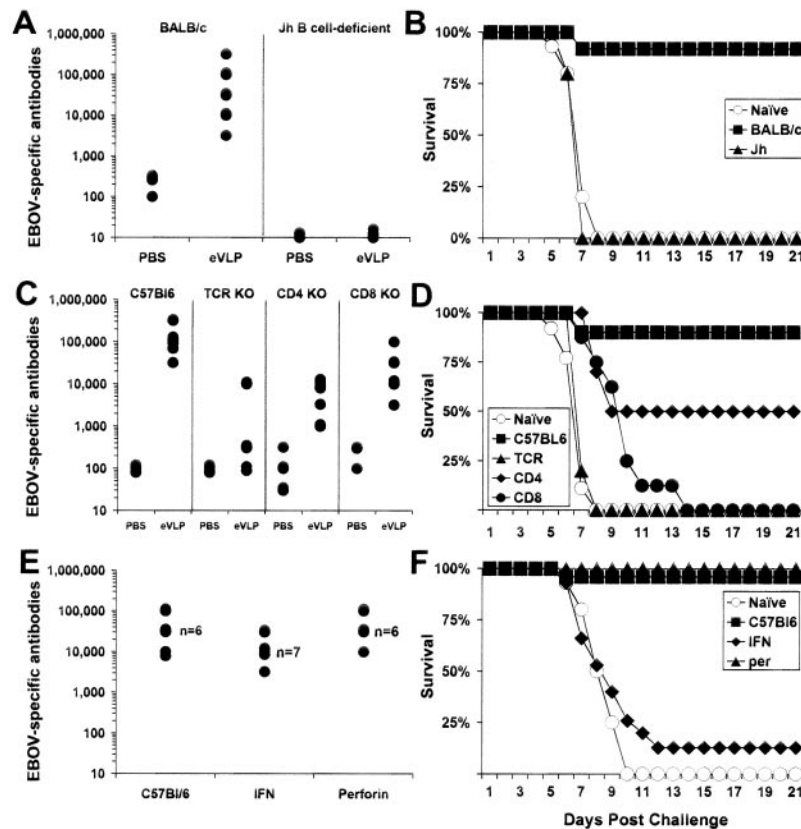


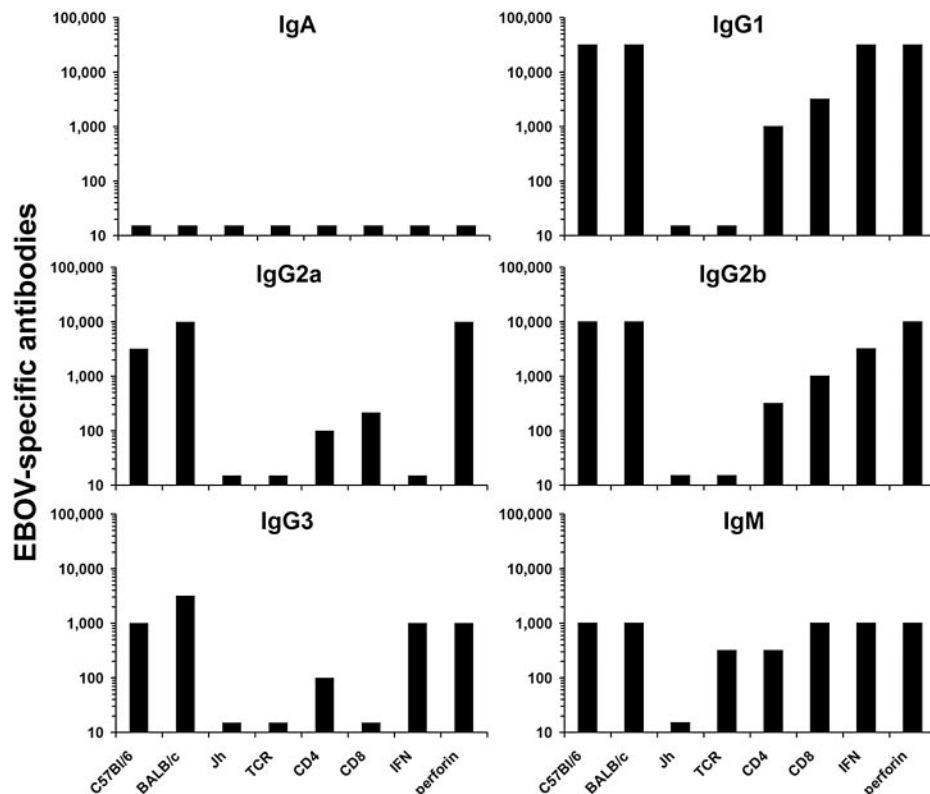
FIGURE 4. Serum Ab responses and protection following vaccination of knockout mice with eVLPs. *A*, Jh B cell-deficient or wild-type BALB/c mice were vaccinated with 10 μ g each of eVLPs and QS-21 or with QS-21 alone twice at 21-day intervals. Total serum anti-EBOV Abs were measured 6 wk after the last vaccination. The results are depicted as the endpoint titers of each mouse (\bullet). These data are representative of two experiments of similar design and outcome. *B*, eVLP-vaccinated Jh B cell-deficient or wild-type BALB/c mice were challenged with 1000 PFU of mouse-adapted EBOV 6 wk after the last vaccination. Results are plotted as percentage survival for each vaccination group ($n = 10$ per group). *C*, $\beta\delta$ TCR, CD4, or β_2m (CD8) T cell-deficient mice or wild-type C57BL/6 mice were vaccinated twice with eVLPs and QS-21 or QS-21 alone. Total serum anti-EBOV Abs were measured by ELISA 6 wk after the last vaccination. The results are depicted as the endpoint titers of the individual mice ($n = 10$). These data are representative of two experiments of similar design and outcome. *D*, Six weeks after the last eVLP vaccination, the T cell or wild-type mice were infected with 1000 PFU of EBOV. Results are plotted as a percentage of survival for each vaccination group ($n = 10$ per group). These data are representative of two experiments of similar design and outcome. *E*, IFN- γ or perforin-deficient mice or wild-type C57BL/6 mice were vaccinated on days 0 and 21 with 10 μ g each of eVLPs and QS-21 or QS-21 alone. Serum samples were collected 6 wk after the last vaccination and the levels of anti-EBOV Abs were measured by ELISA. The endpoint titers of individual mice are shown ($n = 9$ –10). These data are representative of two experiments of similar design and outcome. *F*, IFN- γ or perforin-deficient mice or wild-type C57BL/6 mice were infected with 1000 PFU of EBOV 6 wk after the last vaccination. Results are plotted as a percentage survival for each vaccination group ($n = 13$ –15 per group). These data represent a compilation of data from two individual experiments.

attributed to differences in the route of administration, timing, and dosage (16). EBOV-specific Abs generated by eVLP vaccination may delay viral takeover, but alone appear to be insufficient to protect from lethal infection. This idea is supported by the fact that the CD8⁺ T cell-, CD4⁺ T cell-, and IFN- γ -deficient mice had high titers of EBOV-specific Abs and survived several days longer than adjuvant-vaccinated, control mice (Fig. 4, *D* and *F*). Although CD8⁺ T cell-, CD4⁺ T cell- and IFN- γ -deficient eVLP-vaccinated mice developed strong EBOV Ab responses, the affinity and specificity of the Abs in these knockout mice are unknown and could also contribute to their incomplete protection against EBOV.

Both the eVLP-vaccinated BALB/c and C57BL/6 mice developed CD8⁺ T cell responses to multiple, although different, epitopes within GP and VP40. eVLP-vaccinated BALB/c mice generated GP-specific CD8⁺ responses to a single epitope from amino acids 161–169 (20, 37), whereas C57BL/6 mice generated both CD4⁺ and CD8⁺ T cells specific for a single amino acid sequence within GP_{531–545}. Interestingly, this CD4/CD8 stimulatory peptide WIPYFGPAAEGIYTE contains the putative fusion

peptide sequence in the GP2 portion of the EBOV GP (41). After challenge, all the CD4⁺ and CD8⁺ responses to sequences within GP and VP40 that were detectable before challenge were maintained, and the eVLP-vaccinated BALB/c and C57BL/6 mice also responded to sequences within VP24, VP35, and NP (data not shown). These findings provide evidence that VLP vaccination does not provide sterilizing immunity in mice given that novel immune responses to proteins beyond those used for vaccination are generated following challenge. Several other vaccine strategies have been shown to induce CTL responses against EBOV-specific GP and/or NP in mice, including several epitopes previously identified either before or following EBOV challenge (20, 23–25). The importance of CTL responses was demonstrated by transfer of NP-specific T cells to naive mice, which was sufficient to protect the recipient mice against lethal EBOV infection (23). The significance of these findings in mice is highlighted by studies in humans because patients fatally infected with EBOV do not develop detectable CTL activation, whereas those that survive develop activated CTLs shortly following infection (21).

FIGURE 5. EBOV-specific Ab class and subclass responses following eVLP vaccination of knockout mice. C57BL/6, BALB/c, Jh B cell-deficient (Jh), $\beta\delta$ TCR T cell-deficient (TCR), $CD4^+$ T cell-deficient, β_2m $CD8^+$ T cell-deficient, IFN- γ -deficient, or perforin-deficient were vaccinated with 10 μ g of eVLPs and 10 μ g of QS-21, 10 μ g of QS-21, or PBS alone twice at 21-day intervals. Serum was collected 6 wk following the last eVLP vaccination. The levels of EBOV-specific IgA, IgM, or IgG subclass Abs in pools of sera from 5–10 individual mice were measured by ELISA. Wild-type or knockout mice vaccinated with QS-21 alone or PBS alone did not develop detectable EBOV-specific Abs (data not shown).



$CD4^+$ T cells do not appear to be absolutely required for full protection against EBOV because eVLP-vaccinated $CD4^+$ T cell-deficient mice were partially protected from EBOV challenge. There was not a clear correlation between total EBOV-specific Ab levels and survival of the VLP-vaccinated $CD4^+$ T cell-deficient mice (data not shown), although the eVLP-vaccinated $CD4^+$ T cell-deficient mice had much lower titers of EBOV-specific IgG2a, IgG2b, and IgG3 when compared with vaccinated C57BL/6 mice (Fig. 5). In contrast to our findings, other studies in mice have shown a requirement for the presence of $CD4^+$ T cells using Ab depletions. Vaccination of mice with liposomes encapsulating irradiated EBOV requires $CD4^+$ T cells (20). Recently, Gupta et al. (42) showed that s.c. injection of mice with mouse-adapted EBOV, which effectively vaccinates mice against an otherwise lethal i.p. challenge with mouse-adapted EBOV, requires a combination of both $CD4^+$ T cells and Abs. Therefore, the role of $CD4^+$ T cells in mediating EBOV immunity is unclear at this time.

Induction of innate immune responses, specifically NK cells, can mediate protection from lethal EBOV infection and the innate protection required NK cytolytic activity mediated by perforin production (43). Recently, Gupta et al. (44) confirmed the importance of perforin in early clearance of EBOV when they showed that cytolytic $CD8^+$ T cell clearance of EBOV is perforin-dependent. In contrast, long-term protection by eVLP vaccination requires production of IFN- γ , although it is unknown whether the lack of protection is due to the physical absence of IFN- γ or another IFN- γ -mediated function. The requirement of IFN- γ production for T cell function in protection from EBOV infection is supported by the observation that the kinetics of death in the IFN- γ -deficient mice were very similar to $CD8^+$ T cell-deficient mice (Fig. 4). Although it should be confirmed in nonhuman primate studies, these data suggest that IFN- γ may be useful as a biomarker for predicting efficacy in clinical vaccine trials.

The immune mechanisms required to confer protection against EBOV remain unclear. Until recently, this uncertainty was due to

lack of an efficacious EBOV vaccine combined with the complex interactions of EBOV and the host immune system (12, 45). Further, limitations intrinsic to each of the animal models along with a lack of immunological reagents, samples, and convalescent animals have hampered examination of the requirements for immunity (12). We have used the mouse model of EBOV to discern the importance of individual immune components for protection against EBOV. Based on studies in knockout mice, both EBOV-specific humoral and $CD8^+$ T cell responses are absolutely required for eVLP-mediated protection from EBOV. Our current findings will be used to identify correlates of immunity in eVLP-vaccinated nonhuman primates, for which protective immunity may likely require more than just the generation of circulating EBOV-specific Ab levels.

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Disclosures

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