Ebola virus-like particles protect from lethal Ebola virus infection

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The filovirus Ebola causes hemorrhagic fever with 70–80% human mortality. High case-fatality rates, as well as known aerosol infectivity, make Ebola virus a potential global health threat and possible biological warfare agent. Development of an effective vaccine for use in natural outbreaks, response to biological attack, and protection of laboratory workers is a higher national priority than ever before. Coexpression of the Ebola virus glycoprotein (GP) and matrix protein (VP40) in mammalian cells results in spontaneous production and release of virus-like particles (VLPs) that resemble the distinctively filamentous infectious virions. VLPs have been tested and found efficacious as vaccines for several viruses, including papillomavirus, HIV, parvovirus, and rotavirus. Herein, we report that Ebola VLPs (eVLPs) were immunogenic *in vitro* as eVLPs matured and activated mouse bone marrow-derived dendritic cells, assessed by increases in cell-surface markers CD40, CD80, CD86, and MHC class I and II and secretion of IL-6, IL-10, macrophage inflammatory protein (MIP)-1α, and tumor necrosis factor α by the dendritic cells. Further, vaccinating mice with eVLPs activated $CD4^+$ and $CD8^+$ T cells, as well as $CD19^+$ B cells. After vaccination with eVLPs, mice developed high titers of Ebola virus-specific antibodies, including neutralizing antibodies. Importantly, mice vaccinated with eVLPs were 100% protected from an otherwise lethal Ebola virus inoculation. Together, our data suggest that eVLPs represent a promising vaccine candidate for protection against Ebola virus infections and a much needed tool to examine the genesis and nature of immune responses to Ebola virus.

The filoviruses Ebola (EBOV) and Marburg (MARV) cause severe hemorrhagic fevers in humans and nonhuman primates, which can have very high (70–80%) mortality rates (1). Previous outbreaks have been geographically restricted, but the potential exists for more widespread outbreaks because of the current ease of world travel. Natural outbreaks and laboratory studies indicate the potential for aerosol transmission of these viruses (2–5). A vaccine against filoviruses is needed that protects against a natural or nosocomial outbreak, laboratory accident, or malevolent airborne attack. As of now, there are no available vaccines and no immunological or pharmacological therapies, in part, because of limited knowledge of the mechanisms of filovirus pathogenesis and immunity (6).

Subunit, DNA, and vector-based vaccines have been used with success in rodent models of protection from filovirus infection (7–13). Some of these vaccine strategies are efficacious in nonhuman primates against MARV but not EBOV (8, 10). In contrast with previous disappointments involving several vaccine approaches in nonhuman primates (6, 14, 15), sequential administration of a DNA vaccine and $>10^{10}$ plaque-forming units (pfu) of a defective adenovirus-vectored vaccine or the adenovirus vaccine alone protected nonhuman primates against an EBOV challenge (16–18). Although offering an important proof of concept to be pursued, much remains uncertain about the latter strategy, including an acceptable vaccine dose and the impact of prior immunity to the adenovirus used (19). Thus, there is still a need to explore vaccines against EBOV that do not face these challenges or the particular challenges of other vectored, live-attenuated, or killed virus vaccine strategies (6, 14, 15).

Virus-like particles (VLPs) have been generated in insect and mammalian cell expression systems for a number of viruses including rotaviruses, parvoviruses, human papillomavirus, and HIV by ectopic expression of subsets of their respective viral proteins (20–23). These genome-free VLPs are often morphologically similar to the live virus from which they are derived and are highly immunogenic. VLPs are capable of activating cells involved in both innate and adaptive immunity, ultimately generating both humoral and cell-mediated immunity (22, 24, 25). Interestingly, targeting VLPs to immature dendritic cells (DCs) induced maturation and stimulation of these cells (26–30). Mature DCs are a key antigen-presenting cell population that transports antigen to the peripheral lymph nodes to initiate $CD4^+$ and $CD8^+$ T cell responses (31). DCs are also critically involved in generating antibodies, and VLP vaccinations can protect against viral infections and diseases (22, 24, 25). Therefore, VLPs appear to be excellent tools to deliver antigens to vaccinate against viral infections and represent a tool for dissecting viral immunobiology.

Recently, we demonstrated that successful release of EBOV virions depends on the integrity of lipid raft microdomains (32). Assembly of the virus requires localization of EBOV glycoprotein (GP) to lipid rafts through acylation of dual cysteine residues at the C terminus of the transmembrane domain of GP. Additionally, we showed that expression of both GP and VP40 molecules in the human embryonic cell line 293T results in efficient production and release of Ebola VLPs (eVLPs) in a raft-dependent manner (32). Our objective was to evaluate the ability of eVLPs to activate both innate and adaptive immune arms and induce protective immunity against EBOV infection.

Methods

Virus and Cells. Vero E6 cells were used for virus propagation and plaque assay (33), and human embryonic kidney 293T cells were used for VLP production. EBOV Zaire 1995 or Marburg Musoke virus preparations were purified and inactivated by irradiation with 1×10^7 rad as described (7). 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. All samples removed from the BSL 4 laboratory were γ-irradiated before analysis in BSL 2 or 3 laboratories (8, 10, 32).

Mice. Female BALB/c mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development

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Abbreviations: DC, dendritic cell; EBOV, Ebola virus; eVLP, Ebola virus-like particle; GP, glycoprotein; LPS, lipopolysaccharide; MARV, Marburg virus; pfu, plaque-forming unit(s); TNF, tumor necrosis factor; MIP, macrophage inflammatory protein; VLP, virus-like particle; VP, viral protein.

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Center (Frederick, MD) and used at 6–8 weeks. Mice were divided randomly into vaccination groups. All mice were housed in microisolator cages and provided food and water ad libitum. Blood samples were obtained from the retroorbital sinus or lateral tail vein, or by cardiac puncture under anesthesia, and serum was collected and stored at −70°C.

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 *Guide for the Care and Use of Laboratory Animals* (54). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

VLP Preparation. 293T cells were cotransfected with pWRG7077 plasmid vectors encoding for Ebola VP40 and GP (32) by using calcium phosphate or Lipofectamine 2000 (Invitrogen). To purify the VLPs, the cell supernatants were pelleted over a 20% (wt/vol) sucrose cushion and then further purified on a 10–60% sucrose gradient. The gradient fraction containing the VLPs was determined by Western blotting and electron microscopy.

EBOV and VLP Characterization. Sucrose gradient-purified EBOV or VLPs were processed and examined by transmission electron microscopy as described (32). Total protein concentrations of the VLP or virus preparations were determined after lysis in Nonidet P-40 detergent by using a detergent-compatible protein assay (Bio-Rad). For the preparations of inactivated EBOV and MARV used in these studies, 1 mg of virus contained ≈200 million pfu. All virus and VLP preparations used in these studies were <0.03 endotoxin units per mg, as determined by the *Limulus* amebocyte lysate test (BioWhittaker).

Generation and Stimulation of Murine Dendritic Cells. Murine DCs were generated as described (34), with minor modifications. $CD3^-$, $CD4^-$, $CD8a^-$, $B220^-$, $GR\text{-}1^-$, and IA/IE^- DCs and their precursors were obtained from bone marrow of BALB/c mice by negative selection. The DCs and DC precursors were cultured in RPMI medium 1640 containing 10% FBS, 2 mM L-glutamine, 1 mM Hepes, and 0.1 mM nonessential amino acids (referred to as complete RPMI) with 50 ng/ml murine granulocyte/macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml murine IL-4 (PeproTech, Rocky Hill, NJ) for 6 days, and the cytokines were replenished every other day. For stimulations, two million DCs were incubated with 10 ng/ml lipopolysaccharide (LPS), 25 μg/ml poly I:C, 10 μg of inactivated EBOV, or 10 μg of eVLPs or with medium alone for 24 h.

Flow Cytometry. To assess phenotypic changes of murine DCs or lymphoid cells, the cells were stained with FITC-labeled CD4, CD11c, CD19, CD25, CD40, CD43, CD62L, CD69, CD86, or IA^d, phycoerythrin (PE)-labeled CD4, CD8a, CD19, CD80, or H2K, or CyChrome-labeled CD8a antibodies (BD Pharmingen), washed twice, and fixed in 4% paraformaldehyde before analysis. The percent of positive events was determined after collecting 10,000 or 50,000 events (DCs or lymphocytes, respectively), gated based on forward and side scatter for viable lymphocytes per sample by using CELLQUEST software on a FACSort (BD Immunocytometry Systems).

Cytokine Detection. To determine the levels of cytokines in the supernatant of stimulated cells, cytometric bead assays (BD Pharmingen) were used to detect IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IFN-γ, or tumor necrosis factor (TNF)-α according to the manufacturer's instructions. Levels of cytokines were determined in single samples by cytometric bead assay. Macrophage inflammatory protein (MIP)-1α and IFN-γ secretion in the cell-free virus-infected supernatants were tested in duplicate by ELISA according to the manufacturer's instructions (R & D Systems). The coefficient of variation (COV) for the cytometric bead assay ranged from 4–12%, whereas the COV for the ELISAs ranged from 5–16%.

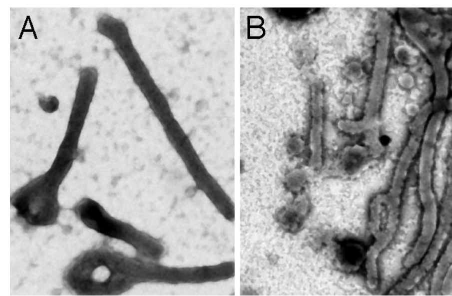

Fig. 1. eVLPs are morphologically similar to authentic filovirus virions. Shown are electron micrographs of EBOV (*A*) or eVLPs (*B*) at ×40,000. Particles, obtained by ultracentrifugation of the supernatants of 293T cells transfected with both Ebola GP and VP40 or VeroE6 cells infected with EBOV, were negatively stained with uranyl acetate to reveal the ultrastructure.

Vaccinations and EBOV Challenge. Mice were vaccinated i.m. or i.p. with 0.1, 1, or 10 μg of eVLPs (n = 5–11) or 10 μg of inactivated EBOV or inactivated MARV (n = 4) diluted in endotoxin-free PBS three times at 3-week intervals, as indicated. Control mice were vaccinated with PBS alone (n = 5 or 10), and mice were challenged 6 weeks after the third vaccination by i.p. injection with 10 or 300 pfu of mouse-adapted EBOV diluted in PBS (35). After challenge, mice were observed twice daily for illness. Vaccine experiments to test protective efficacy after i.m. injections were performed twice, and i.p. examination of VLP efficacy was replicated three times. Serum viremia was determined at day 7 by standard plaque assay as described (33).

To investigate the cellular responses after eVLP vaccination, mice were vaccinated once with eVLPs or PBS alone. Single-cell suspensions of lymphocytes were generated from the spleen, cervical, mediastinal, and mesenteric lymph nodes of individual mice. Phenotypic expression of cells was examined by flow cytometry as described above. Levels of EBOV-specific or plaque-neutralizing antibodies were determined as described (7). The COV for the EBOV-specific antibody ELISA ranged from 8–14%.

Results

eVLPs Mature Bone Marrow-Derived DCs. The eVLPs were purified from supernatants of 293T cells transfected with EBOV GP and VP40. These particles showed remarkably similar morphology to filovirus virions (Fig. 1 *A* and *B*). Therefore, we hypothesized that these genome-free viral particles of EBOV should be a good immunogen and be useful for vaccine purposes. Because DCs represent a critical link between innate and adaptive immune responses and play a crucial role in vaccine immunogenicity, we tested immunogenicity of eVLPs on DCs. Typically, after an encounter with a microbe or microbial products, DCs undergo phenotypic and functional changes, including up-regulation of MHC and costimulatory molecules. These changes enhance the capability of DCs to efficiently present antigens and activate pathogen-specific T cells (31). Bacterially derived compounds such as LPS or structures that mimic viral double-stranded RNA, such as poly I:C, can developmentally transform immature DCs into potent antigen-presenting cells; hence, we used them as controls (27, 36). To evaluate the immunogenicity of the eVLPs, we generated DCs from murine bone marrow cells and exposed them to eVLPs, LPS, or poly I:C for 24 h. eVLP-exposed DCs showed signs of maturation similar to responses induced by LPS

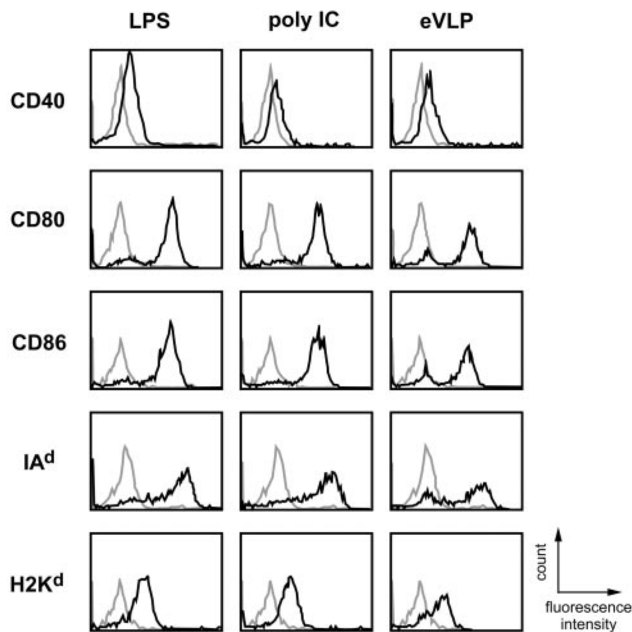

Fig. 2. eVLPs mature murine bone marrow-derived DCs. DCs were stimulated with LPS (10 ng/ml), poly I:C (25 μg/ml), or eVLPs (10 μg/ml) for 24 h. The cells were evaluated for expression of CD40, CD80, CD86, MHC class II (IA^d), or MHC class I (H2K^d) by staining for specific cell surface markers (black trace) or an isotype control antibody (gray trace) and analyzed by flow cytometry. These results represent five experiments of similar design and outcome.

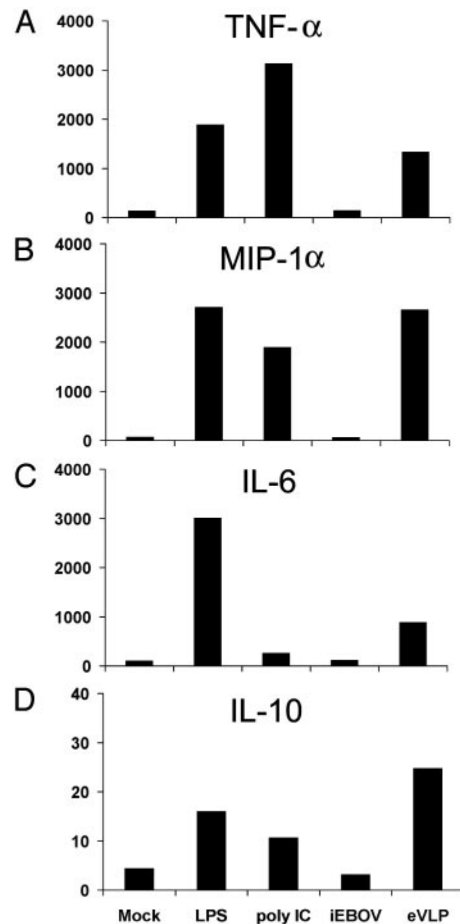

Fig. 3. eVLPs induce chemokine and cytokine secretion in murine DCs. DCs were incubated with medium mock control, LPS (10 ng/ml), poly I:C (25 μg/ml), inactivated EBOV (iEBOV) (10 μg/ml or 2×10^6 pfu equivalents), or eVLPs (10 μg/ml) for 24 h. Cell-free supernatants were assayed for levels (pg/ml) of TNF-α (*A*), MIP-1α (*B*), IL-6 (*C*), and IL-10 (*D*). These results represent five experiments of similar design and outcome.

and poly I:C (Fig. 2). Cell-surface expression of CD40, CD80, CD86, MHC class II marker IA^d, and MHC class I marker H2K^d were up-regulated in DCs incubated with eVLPs. As expected, DCs treated with LPS or poly I:C showed increased cell surface expression of MHC and costimulatory molecules (Fig. 2).

Because it is well established that DCs also secrete a wide array of chemokines and cytokines to attract other immune cells and initiate an immune response (27, 31, 36–38), we conducted cytokine and chemokine analysis of DCs after stimulation with eVLPs. To this end, DCs were treated for 24 h with eVLPs, inactivated EBOV, LPS, or poly I:C, and cell-free supernatants were tested for the presence of cytokines and chemokines. Incubation of DCs with eVLPs induced secretion of proinflammatory chemokines and cytokines such as IL-6, IL-10, MIP-1α, and TNF-α in a similar pattern to LPS and poly I:C (Fig. 3). eVLP stimulation of DCs induced high levels of TNF-α (1,334 pg/ml) and MIP-1α (2,655 pg/ml), intermediate levels of IL-6 (885 pg/ml), and lower amounts of IL-10 (25 pg/ml). Secretion of IL-12p70 and IFN-α was not observed after exposure of the DCs to eVLPs (data not shown). Interestingly, inactivated EBOV induced neither cytokine responses (Fig. 3) nor maturation/activation (data not shown) of the DCs (39). Together, these results indicate that eVLPs are potent activators of DCs and thus could be immunogenic *in vivo*.

eVLP Vaccination Induces Activation of Multiple Cell Types. Effective vaccines require activation of multiple cell types, including B and T cells. To determine whether eVLP vaccination could induce immune responses *in vivo*, BALB/c mice were injected once i.p. with 10 μg of eVLPs. Cellular responses to VLP vaccination were assessed in a number of primary and secondary lymphoid organs, including the spleen, cervical, mesenteric lymph nodes, and Peyer's patches, and compared to aged-matched mock-vaccinated mice injected with PBS alone. Within 3 days, VLP treatment induced significant changes in the activation status of T and B lymphocytes in all lymphoid organs tested (Fig. 4 and data not shown). In the mesenteric lymph node, the percentage of activated lymphocytes was augmented, as assessed by the cell-surface markers CD25, CD43, CD62L, and CD69. Significant increases were observed in the number of CD25$^+$, CD43$^+$, and CD69$^+$/CD4$^+$ T cells (Fig. 4 *A*, *B*, and *D*), as well as in the number of CD25$^+$ and CD69$^+$ CD8$^+$ T cells (Fig. 4 *A* and *D*). CD69$^+$ CD19$^+$ B cells were also significantly increased in numbers in the MLN at 3 days postvaccination (Fig. 4*D*). This cellular activation appeared to be transient, because at early (6–24 h) and late (5–21 days) time points, the increase in activation markers was not as significant (data not shown).

EBOV infection has been associated with massive release of inflammatory chemokines and cytokines late in the course of disease (40–45), either as a cause or effect of pathogenic events. Considering the morphologic similarity of eVLPs to the live virus, serum cytokines were tested at 6 h and 1, 3, 5, 7, 10, 14, and 21 days postvaccination to determine whether eVLPs also induced systemic cytokine release. There was no significant increase in the serum levels of IL-2, IL-4, IL-5, IFN-γ, and TNF-α after a single 10-μg eVLP vaccination as compared to levels in PBS-inoculated mice (data not shown). These data suggest that eVLPs cause no toxic systemic responses, as measured by cytokine release.

Vaccination with eVLPs Induce Humoral Immune Responses in Mice. Protection from EBOV infection is postulated to depend on activation of both arms of the immune response (6). To assess

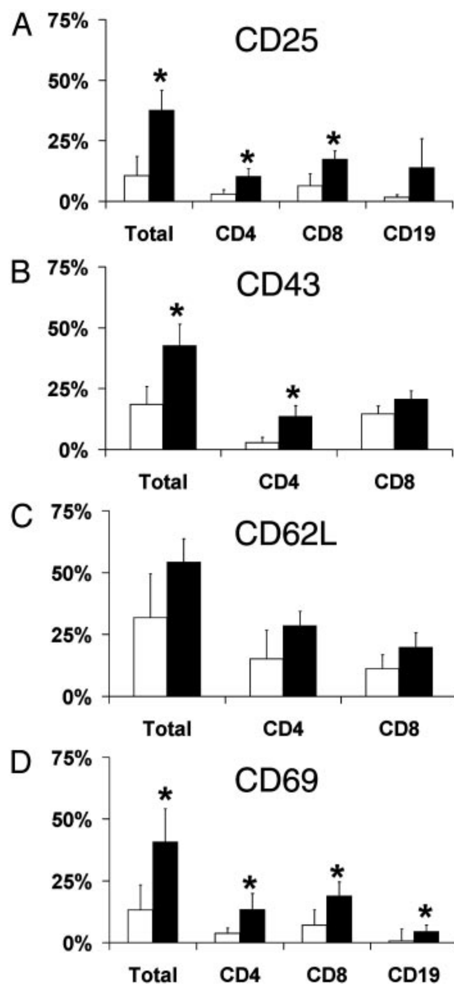

Fig. 4. Activation of multiple cell types in BALB/c mice vaccinated with eVLPs. Lymphocytes were isolated from the mesenteric lymph node of mock- or eVLP-vaccinated mice at 3 days postvaccination. Two million cells each were costained for CD4, CD8, or CD19 and CD25 (*A*), CD43 (*B*), CD62L (*C*), or CD69 (*D*). Percent positive cells were determined by flow cytometry after collection of 25,000 events. Data represent the mean of the percent positive cells from the PBS (unfilled)- or eVLP (filled)-injected mice ($n = 4$) ± SD. Statistical differences ($P \leq 0.05$) in the number of activated cells between PBS- and VLP-vaccinated mice were determined by using a Student's *t* test. *, Statistically significant difference from PBS control.

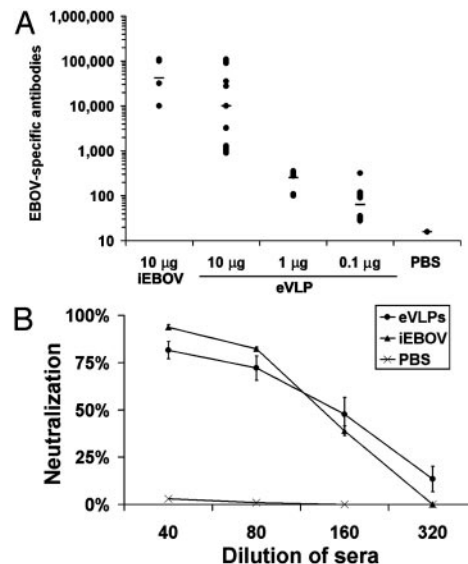

Fig. 5. Serum antibody responses to eVLPs. (*A*) Mice were vaccinated with 0.1, 1, or 10 μg of eVLPs or 10 μg of inactivated EBOV three times at 21-day intervals. Total serum anti-EBOV antibodies were measured 5 weeks after the last vaccination. The results are depicted as the endpoint titers of the individual mice (circles). The horizontal line in each column represents the geometric mean titer for that group of vaccinated mice ($n = 4$–21). (*B*) Percent neutralization of EBOV infection in VeroE6 cells by serum from vaccinated mice. Two-fold dilutions of serum were tested for their ability to neutralize EBOV infection and are plotted as the mean of the percent neutralization for each group of immune sera as compared to mock-treated VeroE6 cells. Error bars indicate the SD of each group.

whether the eVLPs could induce antibody responses specific for EBOV, mice were vaccinated three times i.p. with 0.1, 1, or 10 μg of VLPs, or 10 μg of inactivated EBOV as control, and levels of EBOV-specific serum antibodies were determined by ELISA 5 weeks after the last vaccination. Mice vaccinated with eVLPs developed high titers of EBOV-specific antibodies in a dose-dependent manner (Fig. 5*A*). Similar titers were observed against EBOV when mice were vaccinated with 10 μg of eVLPs or inactivated EBOV (Fig. 5*A*). Additionally, serum from eVLP- or inactivated EBOV-vaccinated mice, but not PBS-vaccinated mice, was able to neutralize filovirus infection of VeroE6 cells as determined by reduction in pfu (Fig. 5*B*). Endpoint titers based on 80% reduction in pfu were similar between both eVLP- (range 40–160) and inactivated EBOV-vaccinated (range 40–160) mice.

eVLPs Protect Mice Against Challenge with Mouse-Adapted EBOV. Because our data demonstrated the ability of eVLPs to induce both humoral and cellular immune responses, we sought to examine whether this immune response could protect mice from lethal challenge with EBOV. BALB/c mice were vaccinated three times at 3-week intervals with eVLPs and challenged with mouse-adapted EBOV 6 weeks after the last vaccination. When eVLP-vaccinated mice were challenged with 10 pfu (≈300 LD_{50}) of mouse-adapted EBOV, we observed survival in a manner dependent on the vaccination dose of eVLPs (Table 1). Mice vaccinated i.p. or i.m. with 10 μg of eVLPs exhibited 100% survival (Table 1) and no morbidity (data not shown). In contrast, i.p. vaccination with 0.1 or 1 μg of eVLPs elicited little to no protective effect (Table 1).

To determine whether VLP vaccination could protect from a high challenge dose, mice were vaccinated i.p. with 10 μg of eVLPs and challenged with 300 pfu (≈9,000 LD_{50}) of mouse-adapted EBOV 6 weeks after the last vaccination (Fig. 6). eVLP-vaccinated mice were 100% protected and showed no sign of illness. Only 25% of mice vaccinated three times with 10 μg of inactivated EBOV were protected from an EBOV challenge (Fig. 6). As expected, inactivated MARV-vaccinated mice were not protected. In this experiment, EBOV infection killed 90% of mice mock-vaccinated with PBS. Unlike the PBS-vaccinated mice, eVLP-vaccinated mice had no detectable viremia at 7 days postchallenge (data not shown). Those surviving the challenge at 7 days were monitored for an additional 21 days. There was no delayed lethality or emergence of symptoms in any of the surviving mice; therefore, mice vaccinated with eVLPs were completely protected from a lethal EBOV challenge.

Discussion

We recently showed that upon transfection of mammalian cells with plasmids expressing the EBOV GP and the VP40 matrix protein, these proteins self-assemble into particles that are morphologically and antigenically similar to EBOV, but the eVLPs do not contain the viral genes necessary for replication

Table 1. Intramuscular and intraperitoneal vaccination with eVLPs

Vaccine	Dose, μg	Route of vaccination*	Geometric mean titer†	Survivors/total‡	Mean time to death§ c, days
eVLPs	10	i.m.	100	5/5	—
eVLPs	10	i.p.	1,412	10/10	—
eVLPs	1	i.p.	251	1/10	6.22 ± 0.44
eVLPs	0.1	i.p.	63	0/10	6.5 ± 1.57
PBS	—	i.p.	16	0/15	6.3 ± 0.73

*Administered on days 0, 21, and 42.
†Geometric mean titer of EBOV-specific antibodies as measured by ELISA.
‡After challenge with 10 pfu of EBOV 6 weeks after the last vaccination.
§Mean time to death ± SD after EBOV challenge.

(32). In this study, we found that eVLPs were highly immunogenic, both *in vitro* in murine DCs and *in vivo* in VLP-vaccinated mice. We demonstrated that vaccination with eVLPs was able to activate both arms of the adaptive immune responses, as demonstrated by the induction of activated B cells and development of Ebola-specific serum antibodies, as well as activation of $CD4^+$ and $CD8^+$ T cells, in eVLP-vaccinated mice. Most importantly, eVLPs were able to protect all mice lethally challenged with EBOV, an effect generally attributed to the combined actions of EBOV-specific antibodies and cellular responses (8, 15, 17, 46, 47).

DCs perform a central role in initiation of both primary and adaptive immune responses (31, 37, 38). DCs fulfill this role by undergoing a maturation process that renders them capable of activating effector cells by means of cytokine secretion and direct cell–cell contact (31, 37, 38). Our findings show that eVLPs efficiently induced DC maturation, activation, and secretion of cytokines and chemokines. This is particularly important in view of recent findings showing that live viral infection with EBOV or MARV, as well as irradiated EBOV virus, does not cause maturation of human DCs (39, 48). Monocytes, macrophages, and DCs appear to be the early targets of filovirus infections (39, 49, 50). Impaired DC maturation may, in part, explain the failure of infected individuals to mount a protective immune response to EBOV infection. There are many differences between a live virus infection and exposure to VLPs. Some of the viral proteins may be virulence factors. For example, EBOV VP35 can interfere with IFN-α secretion (48, 51) and is a candidate for EBOV-mediated down-regulation of immune responses. More detailed studies are needed to determine which component(s) of the Ebola virion is responsible for its immunoregulatory properties. Generating eVLPs containing additional EBOV structural proteins will be useful in determining the mechanisms of the immune responses to EBOV infection.

Administering eVLPs to mice was efficient at quickly alerting adaptive immune responses. Although the activation of B and T lymphocytes seemed to be transient, significant numbers of cells showed activation markers, and expansion of these populations was observed in regional lymph nodes. A single vaccination with eVLPs not only induced activation of $CD4^+$ and $CD8^+$ T cells but also of $CD19^+$ B cells, which may reflect the ability of VLPs to efficiently stimulate multiple arms of the immune system, including humoral and cellular responses. Studies in knockout mice are currently underway to determine whether any of these activated cell types alone are critical for the protective efficacy of the VLP vaccine. Also, we are currently examining the induction of virus-specific CTLs by VLP vaccination and their requirement for vaccine efficacy. It is especially critical to determine immune determinants of protection in animal models to predict efficacy of human vaccines, because conventional field trials studies for protective efficacy of EBOV vaccines may not be possible.

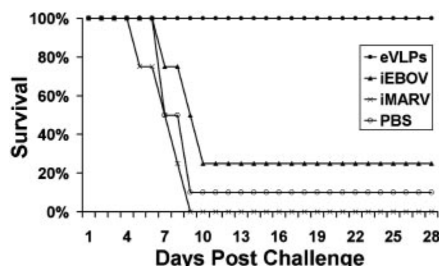

Fig. 6. eVLPs protect mice against EBOV challenge. Mice were vaccinated with 10 μg of eVLPs, inactivated EBOV (iEBOV) or MARV (iMARV), or PBS three times (days 0, 21, and 42), and mice were challenged with 300 pfu of mouse-adapted EBOV 6 weeks after the last vaccination. Results are plotted as percent survival for each vaccination group (n = 4–11 per group).

Our data also demonstrate that eVLPs can induce high titers of EBOV-specific antibody and that these antibodies can inhibit the infection of VeroE6 cells *in vitro*. However, vaccinating mice with eVLPs induced titers of EBOV-specific antibodies similar to vaccination with inactivated EBOV, which provided only marginal protection from Ebola challenge (Figs. 5 and 6). Neither total ELISA antibody titers nor neutralization antibodies based on plaque-reduction assay correlated with protection. This finding appears to be the rule rather than the exception in filovirus vaccine studies (8, 15, 46, 47, 52). Presently, we cannot rule out the possibility that particular types or specificities of anti-EBOV antibodies are most responsible for protection from challenge, as suggested by studies with monoclonal antibodies against EBOV that protected mice from lethal Ebola infection (52). Additionally, it is feasible that antibody is required for protection and that onboard antibody may be an effective host response that delays viral takeover but alone may not be sufficient (53).

eVLPs represent a safe and attractive candidate vaccine. eVLPs lack the viral genome and have no replicative ability and thus deliver the predominant structural antigens without the possibility of infection with EBOV or a carrier vector (6, 12, 16). Additionally, eVLPs are composed of only EBOV GP and VP40 and lack other viral proteins, such as VP35, that are potentially immunosuppressive (51). The absence of a vector in this system rules out the possibility of interference by presence of other unrelated antigens or the chance of previous immunity to the vector itself (16, 17, 19). Another advantage of this technology for vaccination purposes is the ability to easily interchange genes encoding additional filovirus proteins or proteins from multiple filovirus strains into a single VLP. We are currently testing the efficacy of Marburg and Marburg/Ebola hybrid VLPs in mice and guinea pigs. Additionally, the eVLPs are highly immunogenic without adjuvant and could conceivably be used as a vehicle to carry other relevant epitopes or antigens for eliciting protection against other pathogens. The generation of eVLPs provides a useful reagent with many potential applications other than vaccination. Specifically, eVLPs may be used to develop diagnostic reagents for detecting EBOV-infected samples. Gen-

erating VLPs from deletion mutants or chimeric genes will also yield useful information about the requirements for the assembly and budding of filoviruses.

In summary, our data demonstrate the ability of eVLPs to induce both innate and adaptive immune responses that result in protection from EBOV challenge in mice. eVLPs are able to induce EBOV-specific antibodies. The levels of total EBOV-specific and neutralizing antibodies are not indicative of levels of protection in mice. It is likely that the antibodies, in combination with other effector cells including $CD4^+$ and $CD8^+$ T cells, are responsible for protection. eVLPs are effective without the disadvantages of other candidate vaccines such as interference by a vector backbone, previous immunity to the vector, presentation of the antigen in its native form, and, possibly, the absence for the need of adjuvant. Therefore, eVLPs are a promising candidate vaccine, most likely because the critical viral proteins GP and VP40 are presented to the immune system in a conformation similar to wild-type virus without additional virulence proteins. This article provides the groundwork for future studies to evaluate the efficacy of VLPs for both MARV and EBOV in nonhuman primates.

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