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Ebola virus-like particles produced in insect cells exhibit dendritic cell stimulating activity and induce neutralizing antibodies

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Abstract

Recombinant baculoviruses (rBV) expressing Ebola virus VP40 (rBV-VP40) or GP (rBV-GP) proteins were generated. Infection of Sf9 insect cells by rBV-VP40 led to assembly and budding of filamentous particles from the cell surface as shown by electron microscopy. Ebola virus-like particles (VLPs) were produced by coinfection of Sf9 cells with rBV-VP40 and rBV-GP, and incorporation of Ebola GP into VLPs was demonstrated by SDS-PAGE and Western blot analysis. Recombinant baculovirus infection of insect cells yielded high levels of VLPs, which were shown to stimulate cytokine secretion from human dendritic cells similar to VLPs produced in mammalian cells. The immunogenicity of Ebola VLPs produced in insect cells was evaluated by immunization of mice. Analysis of antibody responses showed that most of the GP-specific antibodies were of the IgG2a subtype, while no significant level of IgG1 subtype antibodies specific for GP was induced, indicating the induction of a Th1-biased immune response. Furthermore, sera from Ebola VLP immunized mice were able to block infection by Ebola GP pseudotyped HIV virus in a single round infection assay, indicating that a neutralizing antibody against the Ebola GP protein was induced. These results show that production of Ebola VLPs in insect cells using recombinant baculoviruses represents a promising approach for vaccine development against Ebola virus infection.

Keywords: Ebola; Virus-like particle; Vaccine; Dendritic cell

Introduction

Viruses of four distinct families, arenaviruses, filoviruses, bunyaviruses, and flaviviruses have been found to cause viral hemorrhagic fevers (VHFs), a group of diseases with common symptoms including fever, fatigue, and muscle ache in mild cases and hemorrhage, shock, coma, seizures, and death in severe cases (Borio et al., 2002; Geisbert and Jahrling, 2004). Ebola virus is a member of the filoviridae family and causes severe hemorrhagic fevers in humans and primates with a mortality rate up to 90%, for which no effective treatment or vaccine is available at present (Feldmann et al., 2003). The current outbreak of Marburg virus, another member of the filoviridae family, which has caused infection of almost 400 people with more than 300 fatalities in Angola (WHO, 2005), further emphasizes the urgent need to development an effective vaccine strategy against the Ebola virus as well as the closely related Marburg virus. The immune response that can provide an effective protection against Ebola or Marburg virus infection and pathogenesis has not been clearly defined and accumulated evidence suggests that both antibody and cellular immune responses will be required (Hart, 2003; Nabel, 2003). However, a number of vaccine strategies including inactivated virus, viral protein subunits, DNA, VLPs, as well as recombinant viral vector-based vaccines are under development and have produced promising results (Enserink, 2003; Feldmann et al., 2003; Geisbert and Jahrling, 2003; Hart, 2003). Immunization

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with recombinant viruses or viral replicons expressing Ebola or Marburg proteins has been shown to confer protection against lethal challenge in animal models (Hevey et al., 1998; Pushko et al., 2000; Sullivan et al., 2000, 2003). Although the success of these viral vector-based vaccine strategies provides hope for the containment of emergency outbreaks, the preexisting immune response against these viral vectors may reduce their efficacy in human applications. Moreover, the induction of immune responses against viral vector antigens may limit or preclude the possibility of boosting immunity for obtaining long-lasting protection or the successful vaccination against a different strain should it arise in future. These limitations of viral vector-based vaccines underscore the need for the development of alternative vaccines that can be administered repetitively.

Filoviruses are a family of enveloped, nonsegmented negative-stranded RNA viruses encoding 7 proteins, of which the glycoprotein GP is the only protein that forms the spikes on the surfaces of mature virions and mediates virus entry into the cell (Elliott et al., 1993; Sanchez et al., 1993). The Ebola GP is a type I transmembrane protein which is cleaved into two subunits (GP1 and GP2) by a cellular protease during transport to the cell surface (Sanchez et al., 1998; Volchkov et al., 1998). Infection of cells by Ebola virus leads to assembly and budding of filamentous virus particles (Bavari et al., 2002). The Ebola matrix protein VP40 is the most abundant protein in the virions and has been shown to provide the driving force for the formation of filamentous virus particles (Jasenosky et al., 2001; Noda et al., 2002). Like the matrix proteins or Gag proteins of other enveloped RNA viruses, Ebola VP40 possesses late domains containing the PTAP and PPXY motifs, which interact with the WW domains of cellular proteins such as Nedd4 and TSg101 and play critical roles in the assembly of virus particles (Freed, 2002). It has been shown that expression of Ebola VP40 alone resulted in efficient assembly and budding of Ebola virus-like particles (VLPs) from mammalian cells (Jasenosky et al., 2001; Noda et al., 2002). Furthermore, coexpression of Ebola VP40 and GP proteins results in release of Ebola VLPs in mammalian cell lines (Bavari et al., 2002; Jasenosky et al., 2001; Noda et al., 2002). However, expression of GP alone also results in release of pleiomorphic vesicles containing GP spikes on their surfaces (Noda et al., 2002). VLPs for both Ebola and Marburg viruses produced in mammalian cells have been shown to confer effective protections against lethal challenges in animal models (Swenson et al., 2005; Warfield et al., 2003, 2004), indicating that the VLPs represent a promising vaccine strategy for the control and prevention of filovirus infection and associated hemorrhagic fevers.

VLPs of several viruses have been successfully produced from insect cells using recombinant baculoviruses, and these VLPs have been shown to induce both antibody and cellular immune responses when used to immunize animals (Bertolotti-Ciarlet et al., 2003; Li et al., 1997; Park et al., 2003; Yao et al., 2002). In this study, we investigated the assembly and release of Ebola VLPs from insect cells using recombinant baculoviruses expressing VP40 and GP proteins and characterized the antibody responses induced by these VLP preparations in mice. Our results show that Ebola VLPs produced in insect cells exhibit the ability to stimulate cytokine secretion from dendritic cells, and immunization with these Ebola VLPs can induce neutralizing antibodies against the Ebola GP protein. Production of VLPs in insect cells gives high yields and can be easily adapted for large-scale production, thus representing an attractive approach for the development of an effective vaccine against Ebola virus infection.

Results

Expression of VP40 in insect cells leads to budding and release of EBOV VLPs

We have shown in previous studies that expression of HIV or SIV Gag proteins in insect cells by infection with recombinant baculoviruses leads to assembly and release of VLPs that are morphologically similar to those observed in mammalian cells (Yamshchikov et al., 1995; Yao et al., 2001, 2003). To investigate whether expression of VP40 in insect cells can also lead to assembly of Ebola VLPs, Sf9 insect cells were infected with rBV-VP40 at an MOI of 2 and at 24 h post-infection, the cells were fixed, sectioned, and examined under a transmission electron microscope. As shown in Fig. 1A, infection of Sf9 cells with rBV-VP40 led to formation and budding of filamentous Ebola VLPs from the cell surface as detected by electron microscopy. These filamentous VLPs are about 70 nm in diameter and 800 to 1500 nm in length, which are similar in size and morphology to the virus particles observed in Ebola virus infected cells (Bavari et al., 2002), indicating that the VP40 protein alone is sufficient for assembly of VLPs when expressed in insect cells. Furthermore, as shown in Fig. 1B, filamentous virus-like particles were observed in the VLP preparations from SF9 cells infected with rBV-VP40 and rBV-GP, similar to those observed budding from the cells (Fig. 1A). Similar filamentous particles were also observed in the VLP preparations from the supernatant of Sf9 cells infected with rBV-VP40 alone (not shown). On the other hand, pleiomorphic vesicles that are 70 to 150 nm in diameter were observed in the medium from Sf9 cells infected with rBV-GP alone (Fig. 1C).

Production and characterization of Ebola VLPs produced in insect cells

For large-scale production of VLPs, Sf9 cells (2×10^6/ml) were infected with rBV-VP40 alone or rBV-VP40 and rBV-GP together at an MOI of 2 and 5 respectively, and VLPs were purified in a discontinuous sucrose gradient (10–50%). A visible band between the 30% and 50% sucrose layers was collected, concentrated by ultracentrifugation and then resuspended in PBS giving a final protein concentration of 1 μg/μl. We compared the protein profiles of Ebola VLP preparations from Sf9 cells infected with rBV-VP40 and rBV-GP and the preparations from Sf9 cells infected with rBV-VP40 or rBV-GP alone. VLP preparations from Sf9 cells infected with rBV-Gag (expressing the simian immunodeficiency virus Gag protein) were used as a control. As shown in Fig. 2A, analysis of the

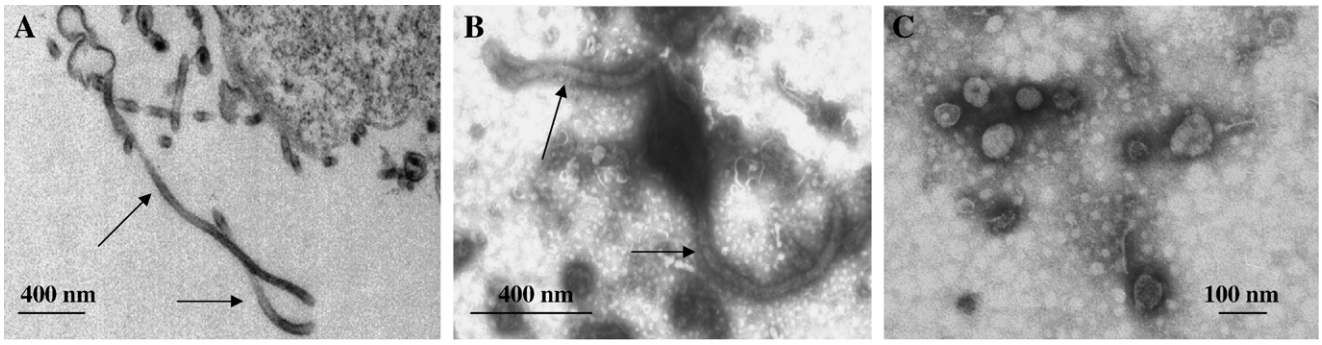

Fig. 1. (A) Budding of Ebola VLPs from insect cells. Sf9 cells were infected with rBV-VP40. At 24 h post-infection, the cells were fixed with 2% glutaraldehyde and then with 2% osmium tetroxide. Thin-section samples were prepared and stained with 1% uranyl acetate followed by examination under a transmission electron microscope. (B) Negative staining of Ebola VLPs. (C) Negative staining of pleiomorphic GP vesicles. Sf9 cells were infected with rBV-VP40 along with rBV-GP (B) or rBV-GP alone (C). At 48 h post-infection, medium was collected, and VLPs or GP vesicles in medium were purified by centrifugation through a 10–50% sucrose gradient and the visible bands containing VLPs or GP vesicles were collected, concentrated by centrifugation and resuspended in PBS at a protein concentration of 1 μg/μl. The samples were stained with 1% uranyl acetate followed by examination under a transmission electron microscope. Arrows indicate elongated VLPs in panels A and B.

protein profile by SDS-PAGE followed by Western blot using a mixture of monoclonal antibodies against VP40 and GP proteins showed that both VP40 and GP are present in the VLP preparations produced by coinfection of Sf9 cells with rBV-VP40 and rBV-GP. As expected, only the VP40 protein or GP protein was detected in the preparations from cells infected with rBV-VP40 or rBV-GP alone respectively. The Ebola GP protein expressed in mammalian cells is synthesized as a precursor and then cleaved into two subunits, GP1 and GP2, which are linked by a disulfide bond (Sanchez et al., 1998; Volchkov et al., 1998). The antibody against GP is specific for the GP1 subunit. To investigate whether the GP proteins in the VLP preparations produced in insect cells are cleaved, we compared the mobility of GP protein in VLP preparations in the presence or absence of the reducing reagent β-mercaptoethanol. As shown in Fig. 2B, the GP protein exhibited a faster mobility by SDS-PAGE after treatment with β-mercaptoethanol, indicating that it was efficiently cleaved in insect cells. Protein profiles for VP40-GP and GP VLPs were also examined by SDS-PAGE and Coomassie blue staining. As shown in Fig. 2C, VP40 proteins are readily detected in VP40-GP VLPs. However, GP proteins were not detected in either VLP preparation, probably due to the lower relative levels of GP in VLPs and/or inefficient staining of glycoproteins by Coomassie

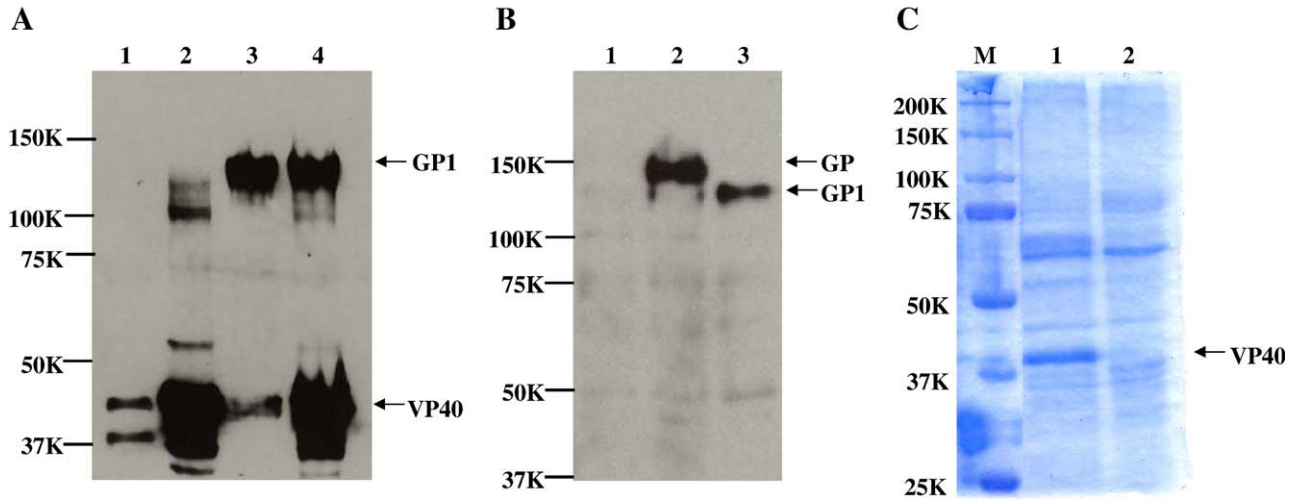

Fig. 2. (A) Western blot analysis of Ebola VLP preparations. Sf9 cells were infected with rBV-SIV-Gag (control VLP, lane 1); rBV-VP40 (lane 2); rBV-GP (lane 3); or rBV-VP40 and rBV-GP (lane 4). The VLP preparations were resuspended in PBS at the concentration of 1 μg/μl and 5 μg of VLP preparations were mixed with reducing protein sample buffer, heated at 95 °C for 5 min, and then subjected to SDS-PAGE followed by Western blot analysis using a mixture of monoclonal antibodies against Ebola VP40 and GP proteins. (B) Mobility of the GP protein in SDS-PAGE in the presence or absence of the reducing reagent β-mercaptoethanol. Ebola VP40-GP VLPs (5 μg) were mixed with reducing (with β-mercaptoethanol, lane 3) or nonreducing (without β-mercaptoethanol, lane 2) protein sample buffers, heated at 95 °C for 5 min, and then subjected to SDS-PAGE followed by Western blot analysis using a monoclonal antibody against the Ebola GP protein. SIV-Gag VLPs mixed with nonreducing protein sample buffer were used as controls (lane 1). (C) Coomassie blue staining of Ebola VLP preparations. 5 μg of VLP preparations was mixed with reducing protein sample buffer, heated at 95 °C for 5 min, and then subjected to SDS-PAGE followed by Coomassie blue staining. Lane M, molecular weight marker; lane 1, VP40-GP VLPs; lane 2, GP vesicle preparations.

blue. A protein band about 60Kd in molecular weight was also detected by Coomassie blue staining in both VP40-GP and GP VLPs, which is probably baculovirus glycoprotein GP64 based on its apparent molecular weight.

To demonstrate that the GP proteins are incorporated into the VLPs, 5 μg of VLP preparations from the supernatant of Sf9 cells infected with rBV-VP40 and rBV-GP as well as rBV-VP40 or rBV-GP alone was loaded on top of a discontinuous sucrose gradient (10–50%) followed by centrifugation at 30K RPM for 1 h in a Beckman SW41 rotor. Fractions were collected, and the proteins in these fractions were concentrated and analyzed by SDS-PAGE and Western blot. As shown in Fig. 3, the majority of both VP40 and GP in the VLP preparation from rBV-VP40 and rBV-GP infected cells cosedimented in fractions 3 to 6. Similarly, the majority of VP40 in the VLP preparation from rBV-VP40 infected cells was also detected in fractions 3 to 6. On the other hand, the majority of GP from the rBV-GP infection was detected in fractions 5 to 7. These result showed that the majority of the released GP proteins from cells expressing both VP40 and GP proteins colocalized with the VP40 proteins in the sucrose gradient and sedimented at a higher density compared with the released GP proteins from cells infected with rBV-GP alone, indicating that the GP proteins are incorporated into VLPs formed by the VP40 proteins.

Stimulation of cytokine secretion from dendritic cells (DCs) by Ebola VLPs

To determine whether Ebola VLPs produced in insect cells are able to stimulate cytokine secretion by DCs, more DCs were incubated with VP40-GP VLPs, VP40 only VLPs, GP vesicle preparations, as well as LPS (positive control) or medium only (negative control) for 24 h, and the amounts of cytokines secreted into the medium were determined by ELISA. As shown in Fig. 4, incubating DCs with Ebola VP40-GP VLPs induced secretion of cytokines and chemokines such as IL-6, IL10, IL-12, and TNF-alpha. The levels of IL-6 and TNF-alpha induced by VP40-GP VLPs are similar to those induced by LPS, while the level of IL-12 is higher, and the level of IL-10 is lower than the levels induced by LPS, respectively. These results indicate that the Ebola VLPs produced in insect cells exhibited the biological activity of DC-stimulation. Furthermore, incubating DCs with preparations from Sf9 cells infected with rBV-GP alone (which consist of pleiomorphic GP vesicles as shown in Fig. 1C) also induced secretion of similar levels of TNF-alpha as well as significantly lower levels of IL-12 and IL-6 compared with VP4-GP VLPs, but no IL-10. On the other hand, incubating DCs with VLPs containing VP40 only did not induce secretion of any cytokines at detectable levels. These results demonstrate that the presence of GP in the VLPs is critical for their DC-stimulating activity, and that the pleiomorphic GP vesicles also exhibit DC-stimulating activity although at a reduced level.

Induction of antibody responses by immunization with Ebola VLPs

The immunogenicity of the VLPs produced in insect cells was evaluated by immunization of mice in comparison with the GP only preparations, which also exhibit DC-stimulating activity but at a reduced level compared with VLPs. Mice were immunized intramuscularly 3 times at 4-week intervals with 50 μg of Ebola VP40-GP VLPs or with the pleiomorphic GP vesicles. As shown in Fig. 2A, the GP vesicle preparation contained similar or slightly higher levels of GP proteins than the same amount of VLP preparation. Serum samples were collected at 2 weeks after each immunization and the levels of antibody responses specific for the Ebola virus GP were determined by ELISA using purified Ebola GP1-histag fusion proteins as coating antigens. As shown in Fig. 5, significant levels of antibodies against Ebola virus GP1 proteins were induced after two immunizations with VP40-GP VLPs, and the levels of antibody response were significantly boosted by the third immunization. In contrast, immunization with GP vesicles

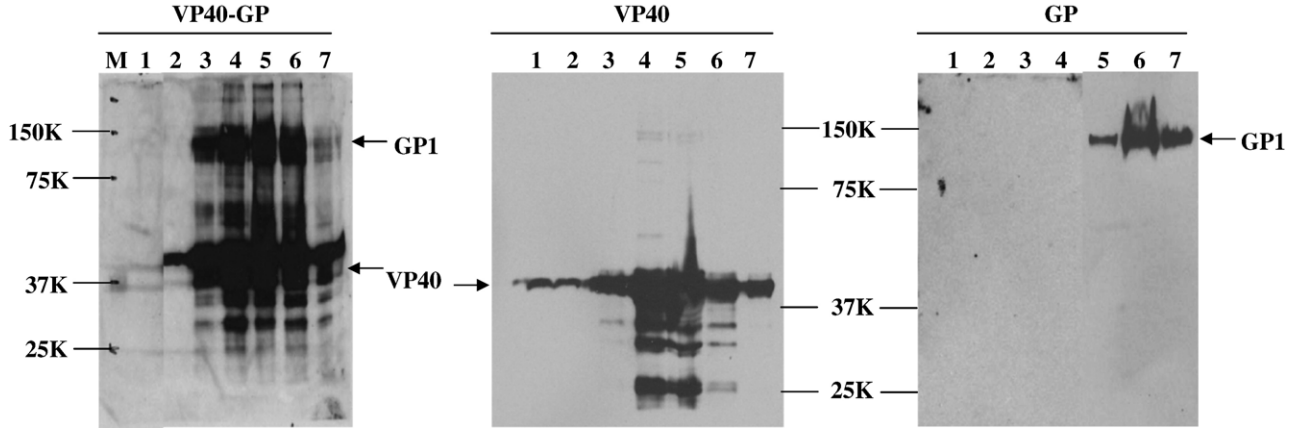

Fig. 3. Distribution of VP40 and GP proteins from different preparations in a sucrose gradient. VLPs or GP vesicles preparations (5 μg each) purified from the supernatant of Sf9 cells infected with rBV-VP40 plus rBV-GP as well as rBV-VP40 or rBV-GP alone were loaded on top of a 10–50% sucrose gradient followed by ultracentrifugation. Six 2-ml fractions (lanes 1 to 6, from the top to the bottom) as well as the pellet (lanes 7) were collected after centrifugation through the sucrose gradient. Proteins in these fractions were concentrated by centrifugation and then analyzed by SDS-PAGE followed by Western blot using a mixture of monoclonal antibodies against Ebola VP40 and GP proteins.

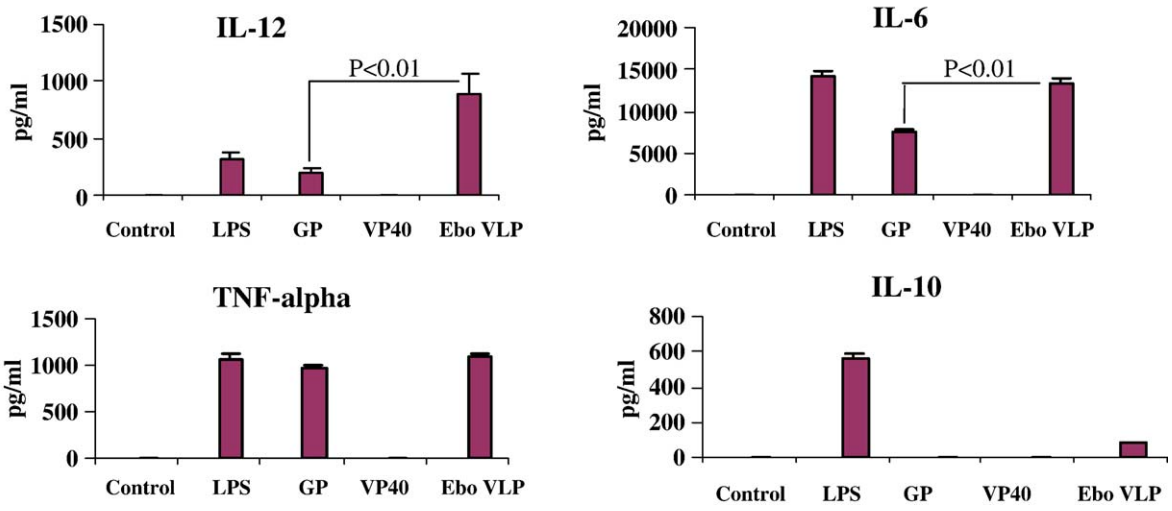

Fig. 4. Ebola VLPs stimulate cytokine secretion by dendritic cells (DCs). Human myeloid DCs were prepared from human PBMC as described in Materials and Methods. DCs were obtained from two healthy donors and incubated with different VLP preparations in duplicates for each DC preparation. DCs incubated with culture medium only were used as negative controls and DCs incubated with LPS (10 ng/ml) were used as positive controls. Cell-free supernatants were harvested 24 h after incubation and assayed for the levels (pg/ml) of TNF-alpha, IL-6, IL10 and IL-12 by ELISA in duplicates. Control, cell culture medium (mock control); LPS, 10 ng/ml (positive control); GP, GP vesicle preparations (10 μg/ml); VP40, VP40 only VLPs (10 μg/ml); Ebo VLP, Ebola VLPs produced in Sf9 insect cells (10 μg/ml). The results shown represent typical results obtained from two different stimulation experiments and error bars represent standard deviations. Significance of statistical differences in the levels of secreted cytokines stimulated by Ebola VLPs or GP vesicle preparations was determined by a *t* test.

preparations only induced significant levels of antibody response after three immunizations, and the levels of antibody response were significantly lower (more than 3-fold) compared with the VP40-GP VLPs. Moreover, it is interesting to note that most of the Ebola GP-specific antibodies induced by VP40-GP VLPs or GP vesicle preparations were of the IgG2a subtype, while the levels of IgG1 antibodies were not significantly higher than those induced in the control group, indicating the induction of a strong Th1-biased immune response.

To assess whether the antibodies induced by VP40-GP VLPs or GP vesicle preparations can neutralize virus infection, we employed a pseudotype virus-neutralization assay adapted from an HIV neutralization assay system as described in our previous studies (Bu et al., 2004; Ye et al., 2004). Env-deficient HIV virus was pseudotyped with Ebola GP protein by cotransfection of 293T cells with a DNA vector expressing Ebola GP and the HIV cDNA, in which a frameshift deletion mutation was introduced in the *env* gene. The ability of the Ebola GP to mediate infection of JC53 cells, a cell line expressing β-gal under the control of the HIV LTR (Derdeyn et al., 2000; Wei et al., 2003), was determined by counting the number of β-gal-expressing cells. Infection of JC53BL cells by GP-pseudotyped virus was completely blocked by hyper-immune mouse sera against Ebola virus at over 1:1000 dilution (data not shown), indicating that the entry of the pseudotyped viruses into the cells is mediated by the Ebola GP protein. As shown in Fig. 6, sera from both VP40-GP VLP- and GP vesicle-immunized mice exhibited a neutralizing activity of about 80% at 1:40 dilution while sera from the control mice did not show significant levels of neutralizing activity against the pseudotyped virus. However, the neutralizing activity of sera from GP vesicle-immunized mice dropped drastically to about 35% at 1:80 dilution and further dropped to about 15% at 1:160 dilution which is not significant over the background levels. In contrast, the neutralizing activity of sera from VP40-GP VLP-immunized mice remained over 50% at 1:160 dilution, which is significantly higher than the neutralizing activity of sera from GP VLP-immunized mice at the same dilution. These results correlate with the levels of GP-specific antibodies induced by VP40-GP VLPs and GP vesicle preparations and show that Ebola VLPs produced in insect cells using the recombinant baculovirus expression system can induce neutralizing antibodies that can prevent Ebola GP protein-mediated virus infection.

Ebola VLPs produced in insect cells or mammalian cells exhibit similar abilities to stimulate cytokine secretion from DCs

It has been reported that Ebola VLPs produced in mammalian cells stimulate cytokine secretion from human DCs (Bosio et al., 2004). The results from the above studies show that Ebola VLPs produced in insect cells also exhibit DC-stimulating activities. It has been noted that protein glycosylation differs between insect and mammalian cells (Jarves and Finn, 1995). We thus produced Ebola VLPs by transfection of 293T cells for direct comparison with Ebola VLPs produced in insect cells. As shown in Fig. 7, similar levels of Ebola GP and VP40 proteins were detected in Ebola VLPs produced in the Sf9 insect cells or mammalian 293T cells. Moreover, the GP protein in Sf9 cell-produced VLPs exhibited a faster migration rate than the GP protein in 293T cell-produced VLPs in SDS-PAGE (comparing lane 2 and lane 3), probably due to differences in glycosylation. We further compared DC-stimulating activities between Ebola VLPs produced in Sf9 insect cells and mammalian 293T cells. As shown in Fig. 8, similar levels of

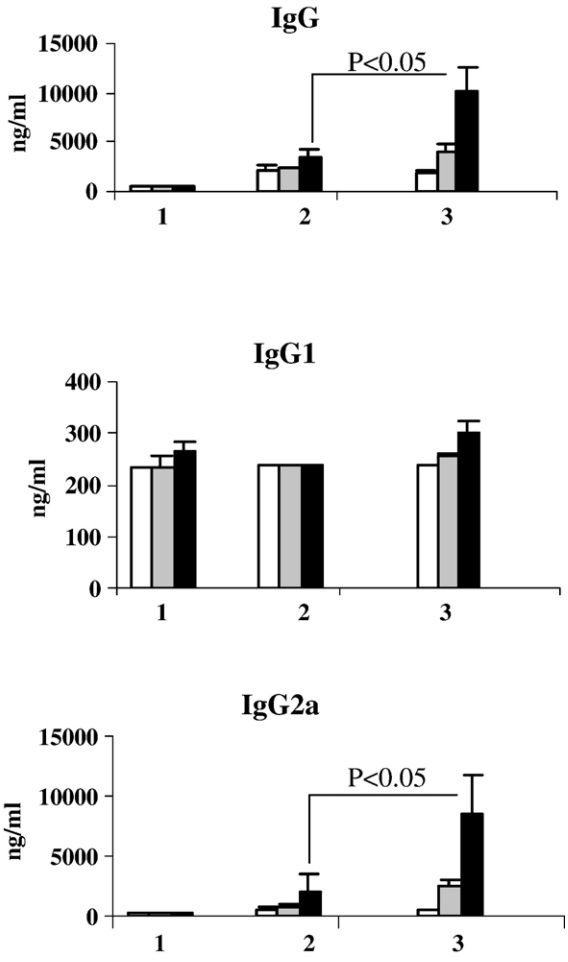

Fig. 5. Antibody responses against Ebola GP protein induced by immunization with VLPs or GP vesicles. Mice (groups of 6) were immunized intramuscularly with 50 μg of VLP preparations three times at 4-week intervals. Serum samples were collected at 2 weeks after each immunization (open bars, after the first immunization; gray bars, after the second immunization; and black bars, after the third immunization) and assayed for Ebola GP specific antibodies by ELISA using purified Ebola GP1 as coating antigen. A standard curve was generated using purified mouse antibodies for each mouse antibody subtype and used for calculation of the equivalent amount of GP-binding antibodies in serum samples. Group 1, immunized with PBS; group 2, immunized with GP preparations; group 3, immunized with VP40-GP VLPs. Error bars represent standard deviations among individual mouse serum samples in each group. Significance of statistical differences in the levels of antibody responses induced by immunization with VP40-GP VLPs or GP vesicle preparations was determined by *t* test.

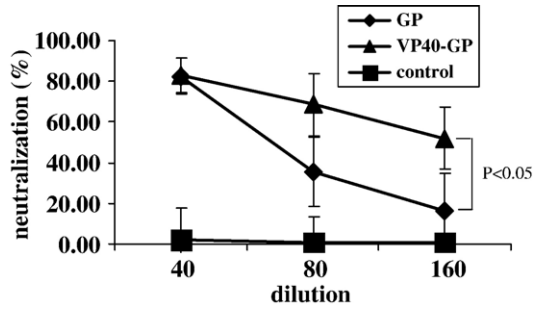

Fig. 6. Neutralization of Ebola GP pseudotyped HIV by sera from immunized mice. Serum samples obtained from mice (groups of 6) immunized with PBS, GP vesicle preparations, or VP40-GP VLPs at two weeks after the third immunization were heat-inactivated and mixed with 100 pfu of Ebola GP-pseudotyped HIV at 1:40, 1:80, or 1:160 dilutions and incubated at 37 °C for 1 h and then added to JC53 cells. At 48 h post-infection, the cells were stained for β-galactosidase expression by addition of X-Gal, and the numbers of blue cells were counted under a light microscope. The percentages of reduction in the number of blue cells in comparison to the control wells were calculated as described in Materials and Methods. Standard deviations for serum samples from each mouse of the group were shown. Significance of statistical differences in neutralizing activity at 1:160 dilution of serum samples from mice immunized with VP40-GP VLPs or GP vesicle preparations was determined by a *t* test.

proinflammatory cytokines such as IL6, IL8, IL12, as well as TNFa were detected in the medium of DCs stimulated with Ebola VLPs produced in Sf9 cells or 293T cells. Moreover, the levels of secreted IL6 and TNFa from DCs stimulated by VLPs produced in Sf9 cells relative to stimulation by LPS were similar to those observed in Fig. 4, despite the use of different batches of VLPs and LPS in these experiments. On the other hand, DCs stimulated with Ebola VLPs produced in mammalian 293T cells secreted higher levels of IL10, an anti-inflammatory cytokine (Pestka et al., 2004). However, whether this difference in IL10 stimulation is due to the differences in glycosylation or other differences in VLPs produced in different cell types remains to be determined. Furthermore, DCs stimulated with Ebola VLPs produced in Sf9 or 239T cells also showed similar upregulation of activation makers such as CD80 and CD83 as detected by FACS analysis (data not shown). Taken together, these results show that Ebola VLPs produced in Sf9 insect cells or mammalian 293T cells contain similar profiles of Ebola GP and VP40 proteins and exhibit similar DC-stimulating activities

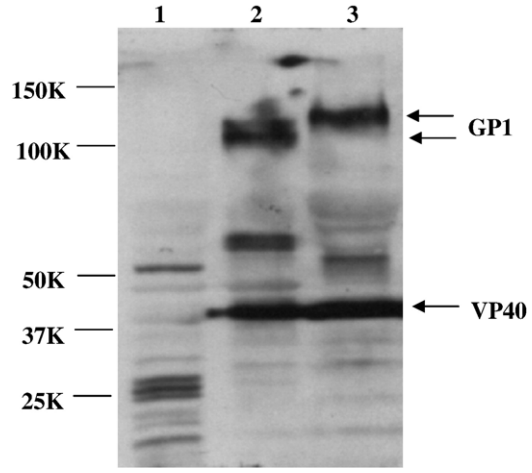

Fig. 7. Western blot analysis of Ebola VLPs produced in Sf9 insect cells and 293T mammalian cells. Ebola VLPs produced in insect cells were carried out by coinfection of Sf9 cells with rBV-VP40 and rBV-GP. Ebola VLPs produced in mammalian cells were carried out by cotransfection of 293T cells with DNA expression vectors pcDNA3-VP40 and pcDNA3-GP. The VLP preparations were resuspended in PBS, and 5 μg of VLP preparations was mixed with reducing protein sample buffer, heated at 95 °C for 5 min, and then subjected to SDS-PAGE followed by Western blot analysis using a mixture of monoclonal antibodies against Ebola VP40 and GP proteins. Lanes 1, SIV Gag VLPs produced in Sf9 cells (control); 2, Ebola VLPs produced in Sf9 insect cells; 3, Ebola VLPs produced in 293T mammalian cells.

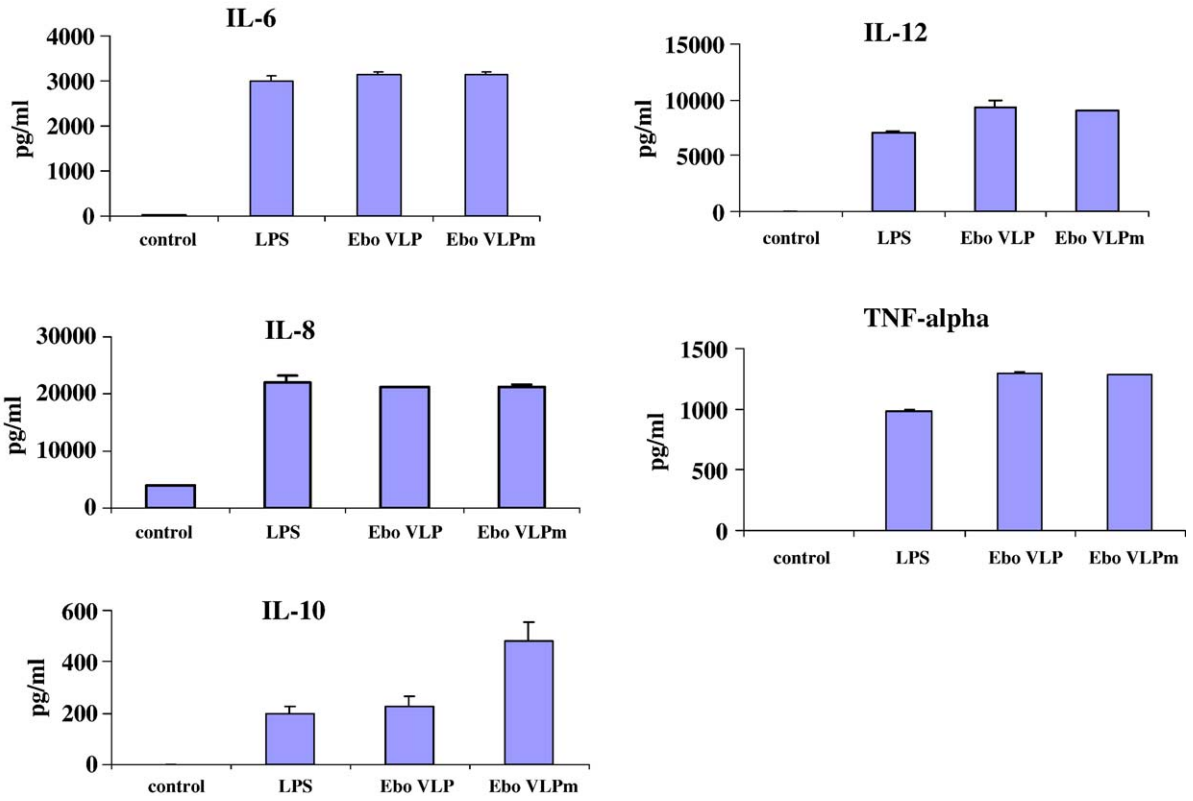

Fig. 8. Ebola VLPs produced in Sf9 insect cells or 293T mammalian cells exhibit similar activities to stimulate cytokine secretion by dendritic cells (DCs). Dendritic cells were prepared from human PBMC and incubated with VLPs produced in Sf9 insect cells or mammalian 293T cells as described in Materials and Methods. DCs were obtained from two healthy donors and incubated with different VLP preparations in duplicates for each DC preparation. DCs incubated with culture medium only were used as negative controls and DCs incubated with LPS (10 ng/ml) were used as positive controls. Cell-free supernatants were harvested 24 h after incubation and assayed for the levels (pg/ml) of IL-6, IL-8, IL10, IL-12, and TNF-alpha by ELISA in duplicates. Control, cell culture medium (mock control); LPS, 10 ng/ml (positive control); Ebo VLP, Ebola VLPs produced in Sf9 insect cells (10 μg/ml); Ebo VLPm, Ebola VLPs produced in mammalian 293T cells (10 μg/ml). The results shown represent typical results obtained from at least two different stimulation experiments and error bars represent standard deviations.

despite the known differences in glycosylation between these two cell types.

Discussion

In this study, we investigated the assembly of Ebola VLPs in insect cells using recombinant baculoviruses. Our results show that expression of VP40 alone or together with GP in insect cells led to efficient assembly and release of VLPs, which exhibit a filamentous structure resembling Ebola virions. On the other hand, expression of Ebola GP alone in insect cells led to release of pleiomorphic vesicles. Previous studies on Ebola VLP assembly and budding in mammalian cells also indicated that the expression of VP40 is essential and sufficient for driving the formation of filamentous particles (Jasenosky et al., 2001; Noda et al., 2002). We observed that by coexpression with VP40, GP was found to cosediment with VP40 in a sucrose gradient, and exhibited a different sedimentation pattern from GP vesicles, indicating that the GP proteins were incorporated into the VLPs formed by the VP40 proteins. Moreover, the GP proteins in VLPs were found to be efficiently processed into the GP1 and GP2 subunits that are linked by disulfide bonds. The yield for purified VLPs produced in Sf9 insect cells was typically between 5 to 10 mg/l of cell culture, which is similar to the yields we consistently obtained for HIV and SIV VLPs (Yamshchikov et al., 1995; Yao et al., 2001). In contrast, we found that production of HIV VLPs in mammalian cells using recombinant vaccinia viruses gave a yield of about 1 mg/l of cell culture (unpublished observations), and it has been reported that the yield for HIV VLP production in a stable cell line is less than 1 mg/l of tissue culture medium (Montefiori et al., 2001).

One of the distinctive properties of Ebola VLPs is their ability to stimulate DCs or macrophages to secret cytokines. It has been reported that Ebola VLPs produced in mammalian cells but not inactivated Ebola virions can stimulate production and secretion of proinflammatory cytokines such as IL-6, IL-8, and TNF-alpha by DCs (Bosio et al., 2004; Warfield et al., 2003). This property of the Ebola VLPs has been suggested to play an important role in eliciting protective immune responses, since immunization with VLPs but not inactivated virions was found to confer effective protection against lethal Ebola virus challenge despite induction of similar levels of neutralizing antibodies in immunized animals (Warfield et al., 2003). In this study, we found that the VP40-GP VLPs produced in insect cells were able to stimulate cytokine secretion by DCs, and the levels of secreted cytokines from DCs stimulated with Ebola VLPs produced in Sf9 insect cells were similar to those from DCs stimulated with Ebola VLPs produced in mammalian 293T

cells. In contrast, the VP40-VLPs formed by VP40 alone did not stimulate any detectable cytokine secretion, indicating that the presence of GP is critical for the observed cytokine stimulation activity. It is interesting to note that glycoproteins synthesized in insect cells lack modification by complex carbohydrates, and the side chains are of the high-mannose type (Mellquist-Riemenschneider et al., 2003). Our results suggest that the glycosylation difference between mammalian and insect cells does not significantly affect the ability of Ebola VLPs to stimulate cytokine secretion by DCs, indicating that the Ebola VLPs produced in insect cells possess similar functional activities to those produced in mammalian cells. It has been reported that, unlike Ebola VLPs, the purified Ebola GP proteins could not stimulate cytokine secretion by macrophages (Wahl-Jensen et al., 2005). However, we observed that the GP vesicle preparations devoid of VP40 were also able to stimulate cytokine secretion by DCs, although at reduced levels. Taken together, these observations suggest that the presentation of Ebola GP proteins in a membrane bound or multivalent form may be required for exhibiting DC-stimulating activity, and the higher DC-stimulating activity observed for VP40-GP VLPs may result from a relatively more ordered arrangement of GP proteins on the membrane surface of VLPs.

Evaluation of immunogenicities in mice showed that both VP40-GP VLPs and GP vesicle preparations induced antibody responses against GP in immunized animals that are mainly of the IgG2a subtype, indicating the induction of strong Th1-biased immune responses. However, immunization with the VP40-GP VLPs induced significantly higher levels of antibody response than the GP vesicle preparation. It has been shown that the glycoprotein of vesicular stomatitis virus presented in a highly repetitive form in virus particles is more potent in inducing B cell response and antibody production than the same antigen presented in a poorly organized form (Bachmann et al., 1993; Bachmann and Zinkernagel, 1996). Therefore, it is possible that the GP proteins in Ebola VLPs are presented in a more ordered arrangement and thus are more potent in eliciting antibody responses as well as in stimulating cytokine secretion by DCs. On the other hand, the higher levels of antibody response induced by VP40-GP VLPs may be due to their enhanced abilities to stimulate DCs, the professional antigen presenting cells. The Ebola GP protein expressed in insect cells has been shown to bind to monoclonal antibodies against different epitopes (Mellquist-Riemenschneider et al., 2003). We observed that both VP40-GP VLPs and the GP vesicle preparations induced neutralizing antibodies that can block virus infection mediated by the Ebola GP protein, indicating that the neutralizing epitopes are preserved in the Ebola GP proteins synthesized in insect cells. Sera from VP40-GP VLP immunized mice neutralized about 80% infectivity of Ebola GP-pseudotyped HIV pseudovirions at 1:40 dilutions and about 50% infectivity at 1:160 dilution. This is similar in range to the neutralizing titers of sera from mice immunized with Ebola VLPs produced in mammalian 293T cell, which were shown to neutralize 50% infectivity of Ebola virus at about 1:100 dilutions as reported by Warfield et al. (2003). In agreement with the levels of GP-specific antibody responses, sera from mice immunized with VP40-GP VLPs exhibited significantly higher levels of neutralizing activities than sera from mice immunized with the GP vesicle preparations.

VLPs for both Ebola and Marburg viruses produced in mammalian cells have been shown to confer effective protection in small animal models against lethal challenge (Swenson et al., 2005; Warfield et al., 2003). Furthermore, a recent study showed that immunization with Ebola VLPs produced in mammalian cells induced both antibody and T cell responses against GP and VP40 proteins, and both humoral and cellular immune responses induced by Ebola VLPs are critical in conferring protection against lethal challenge (Warfield et al., 2005). The results reported in this study showed that Ebola VLPs produced in insect cells exhibit DC-stimulating activities and can induce neutralizing antibodies against the Ebola GP protein similar to VLPs produced in mammalian cells, despite known differences in glycosylation between mammalian cells and insect cells. However, it remains to be determined whether insect-cell-produced Ebola VLPs exhibit similar immunogenicities as mammalian-cell-produced VLPs in eliciting both humoral and cellular immune responses and conferring protection against Ebola virus infection. VLPs can be administered repeatedly to vaccinated individuals and the nonreplicative nature of VLPs and their lack of viral genomic RNA make them safe for broad and repeated application, and they thus represent an attractive vaccine strategy against filovirus infection. Production of Ebola VLPs in insect cells using recombinant baculoviruses gives high yield and the production process can be easily adapted for large-scale manufacture, offering an attractive approach for the development of an effective and economical vaccine strategy to prevent Ebola virus infection.

Materials and methods

Cells and antibodies

Spodoptera frugiperda Sf9 cells were cultured in SF-900 II serum-free medium with penicillin/streptomycin in suspension. Monoclonal antibodies against Ebola VP40 and GP proteins were kindly provided by Dr. Y. Kawaoka.

Generation of recombinant baculoviruses

Recombinant baculoviruses expressing Ebola VP40 or GP proteins were generated following similar procedures as described in our previous studies (Yamshchikov et al., 1995). The cDNAs for Ebola GP and VP40 proteins (Ebola Zaire strain, Mayinga isolate, kindly provided by Dr. P. Rollin, Center for Disease Control and Prevention) were amplified by PCR and cloned into the plasmid vector pBlueScript IIKS under the T7 promoter and confirmed by sequencing. These genes were then cloned into an AcMNPV transfer vector pC/pS1 under the control of a hybrid capsid/polyhedrin promoter (Pcap/polh), and these plasmids were used to generate recombinant baculoviruses rBV-VP40 and rBV-GP expressing Ebola VP40 or GP proteins, respectively. Sf9 cells were

cotransfected with a mixture of the AcMNPV DNA and the baculovirus transfer vector pC/pS1 containing Ebola VP40 or GP genes respectively by using a BacGold Cotransfection Kit (Invitrogen) following the manufacturer's instruction. Recombinant baculoviruses were plaque purified and expanded in Sf9 cells to generate virus stocks, and the titers were determined using a Fastplax Assay Kit (Invitrogen).

Protein expression and VLP production in insect cells

Sf9 cells in suspension cultures were infected with rBV-VP40 or rBV-GP alone or coinfected with rBV-VP40 plus rBV-GP at the MOIs (multiplicity of infection) of 2 for rBV-VP40 and 5 for rBV-GP respectively. Media were harvested 48 h post-infection and clarified by centrifugation at 6000 rpm for 30 min. Supernatants were ultracentrifuged at 28,000 rpm for 1 h, the VLPs in the pellets were resuspended in PBS and further purified on a discontinuous 10–50% (w/v) sucrose gradient. A visible band between the 30% and 50% sucrose layers was collected, concentrated by centrifugation and then resuspended in PBS at a final protein concentration of 1 μg/μl. The presence of VP40 and GP proteins in the purified preparations was analyzed by SDS-PAGE followed by Western blot using antibodies against Ebola viral proteins or Coomassie blue staining.

Examination of EBOV VLP by electron microscopy

For EM study of VLP budding from insect cells, Sf9 cells were infected with rBV-VP40 at an MOI of 2. At 24 h post-infection, the cells were fixed with 2% glutaraldehyde and then with 2% osmium tetroxide. Thin-section samples were prepared and examined with a transmission electron microscope. For EM study of purified VLPs, VLP preparations from Sf9 cells coinfected with rBV-VP40 plus rBV-GP or with rBV-GP or rBV-VP40 alone were stained with 1% uranyl acetate and then examined under a Hitachi-H7500 transmission electron microscope.

Production of Ebola VLPs in 293T cell

The genes for Ebola VP40 and GP proteins were cloned into the plasmid vector pcDNA3 under the CMV promoter. 293T cells were seeded in T175 flasks over night and then transfected with pcDNA3-VP40 and pcDNA3-GP using Polyfect (Qiagen) following manufacture's protocols. At 48 h post-transfection, the cell supernatant was collected, and VLPs were purified by centrifugation through a 20% sucrose cushion at 30K RPM in a Beckman SW41 rotor. VLPs were resuspended in PBS at about 0.5 μg/μl for characterization by Western blot as well as stimulation of DCs.

Stimulation of dendritic cells and detection of cytokine secretion

Human myeloid dendritic cells were prepared from human PBMC as described in our previous studies (Agrawal et al., 2003; Mahanty et al., 2003). Briefly, PBMC were obtained by aphresis from healthy human donors. Monocyte-derived DC was generated by culturing purified monocytes (Ficoll, and CD14 MACs beads, Miltenyi Biotech) in RPMI 1640 with 10% FBS for 6 days in the presence of GM-CSF (100 ng/ml) and IL-4 (5 ng/ml). This resulted in enrichment for CD11c+ CD1a +HLA-DR+ MDDC (>90%). On day 6 of culture, 0.5×10^6/ml of immature human myeloid DCs from two different donors were each stimulated in vitro with 10 μg of each respective VLP preparations as indicated in figure legends (Ebola GP, GP/VP40, VP40, or Ebola VLPs produced in mammalian 293T cells), as well as with LPS (10 ng/ml, positive control), or with untreated media (negative control) in 12-well plates in duplicates. Supernatants were harvested after 24 h of incubation at 37 °C in 5% CO_2. Cytokine levels in cell supernatants were measured by ELISA in duplicates using commercially available kits (BD Pharmigen, R&D) for IL-6, IL-8, IL-10, IL-12, as well as TNF-alpha according to the manufacturer's instruction. The stimulation experiments were repeated once to ensure consistency of obtained results. The results shown represent typical results obtained from two different stimulation experiments and error bars represent standard deviations. Significance of statistical differences in the levels of secreted cytokines stimulated by VP40-GP VLPs or GP vesicle preparations was determined by a *t* test.

Immunization of mice and sample collection

Female BALB/c mice (8 weeks old) were obtained from Charles River Laboratory and housed at the Emory University Animal Facility in micro-isolator cages. Groups of mice (six per group) were immunized with a total of 50 μg of indicated purified VLP preparations per mouse by intra-muscular injection with 25 μl VLP preparation per side in both side quadriceps, followed by boosting with the same dose of VLPs at weeks 4 and 8. Blood samples were collected from the retro-orbital sinus under anesthesia at 1 week prior to the first immunization and 2 weeks after each immunization, and serum samples were collected and stored at −80 °C until further analysis.

ELISA

The coding sequence for the Ebola virus GP1 subunit was linked in frame to the 5′ of a DNA segment encoding for 6 Histidines followed by a stop codon. The gene for the GPI-histag fusion protein was cloned into the transfer vector pRB21 for generation of recombinant vaccinia viruses expressing the Ebola GP1-histag fusion protein. His-tagged GP1 proteins were expressed in HeLa cells by infection with the recombinant vaccinia virus and purified using Ni-NTA agarose beads (Qiagen). Ebola virus-specific antibodies were measured in individual mouse serum samples by an enzyme-linked immunoabsorbent assay (ELISA) using purified GP1-histag proteins as coating antigens. Briefly, the assays were performed in 96-well polystyrene microtiter plates (Nunc) coated overnight at 4 °C with purified GP1-histag proteins at a concentration of

2 μg/ml. Serial dilutions of serum samples were incubated at RT for 2 h on coated and blocked ELISA plates, and the bound immunoglobulins were detected with horseradish peroxidase-labeled goat antibodies against mouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates). The wells were developed with TMB (Sigma). The color reaction was stopped with hydrochloric acid (0.2N) and the absorbance at 450 nm was read in an EL312 Bio-Kinetics microplate reader (Bio-Tek Instruments Inc., Winooski, VT). A standard curve was constructed by coating each ELISA plate with serial 2-fold dilutions of purified mouse IgG, IgG1, or IgG2a with known concentrations, respectively, and the concentrations of Ebola GP-specific antibodies in serum samples were calculated using obtained standard curves and expressed as the amount of antigen-specific antibody in 1 ml of serum samples (ng/ml).

Neutralization assay

Neutralizing antibodies against Ebola GP were analyzed using a single-round infectivity assay adapted from an assay system used in our previous studies of HIV (Bu et al., 2004; Ye et al., 2004). This assay is based on an indicator cell line, JC53-BL, which is a derivative of HeLa cells and contains reporter cassettes of β-galactosidase under an HIV-1 LTR that is activated by expression of the HIV Tat protein (Derdeyn et al., 2000; Wei et al., 2003). The Ebola GP pseudotyped HIV was prepared by cotransfection of 293T cells with DNA vectors for the HIV backbone and the Ebola GP and the titer of the pseudotyped virus was determined in the JC53BL cells. Typically a titer of approximately 10^5 pfu (blue plaque forming units) per ml was obtained. For analysis of neutralizing antibodies, dilutions of heat-inactivated serum samples were incubated with 100 pfu of pseudotyped virus in a 96-well plate for 1 h at 37 °C and then added to JC53BL cells in the presence of DEAE-dextran and incubated for 2 days at 37 °C. Cells were then stained for β-galactosidase expression. Neutralization was calculated as the percentage of reduction of the number of blue cells in sample wells, which were infected with pseudotyped virus incubated with serum samples, in comparison to the control wells, which were infected with the same dose of pseudotyped virus incubated with the same dilution of group-matched preimmune serum samples [(the number of blue cells in control wells-the number of blue cells in sample wells)/(the number of blue cells in control wells) × 100%].

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