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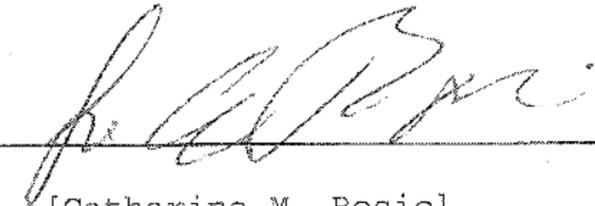
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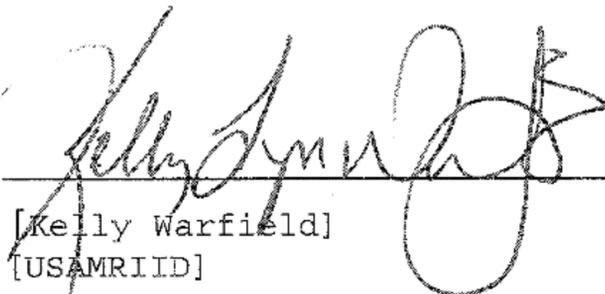

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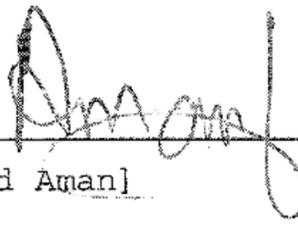
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Ebola and Marburg virus-like particles activate human myeloid dendritic cells

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Abstract

The filoviruses, Ebola (EBOV) and Marburg (MARV) viruses are potential global threats and cause deadly hemorrhagic fevers. Although, both EBOV and MARV exponentially replicate in dendritic cells (DCs), these viruses do not elicit cytokine secretion and fail to activate and mature infected DCs. Here, we employed virus-like particles (VLP) of EBOV and MARV to investigate whether these genome-free particles maintain similar immune evasive properties as native filoviruses. Confocal microscopy indicated that human myeloid-derived DCs readily took up the VLPs. However, unlike EBOV and MARV, the VLPs induced changes in DCs to include, upregulation of co-stimulatory molecules (CD40, CD80 and CD86) as well as MHC receptors and CD83. VLPs also elicited secretion of a variety of pro-inflammatory cytokines, specifically TNF- α , IL-8, IL-6 and MIP-1 α . Thus, our findings suggest that unlike EBOV and MARV, the VLPs are effective stimulators of DCs and thus may show strong potential in enhancing innate and specific adaptive immune responses.

Ebola and Marburg virus-like particles activate human myeloid dendritic cells

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Abstract

The filoviruses, Ebola (EBOV) and Marburg (MARV), are potential global health threats, which cause deadly hemorrhagic fevers. Although both EBOV and MARV logarithmically replicate in dendritic cells (DCs), these viruses do not elicit DC cytokine secretion and fail to activate and mature infected DCs. Here, we employed virus-like particles (VLPs) of EBOV and MARV to investigate whether these genome-free particles maintain similar immune evasive properties as authentic filoviruses. Confocal microscopy indicated that human myeloid-derived DCs readily took up VLPs. However, unlike EBOV and MARV, VLPs induced maturation of DCs including upregulation of costimulatory molecules (CD40, CD80, CD86), major histocompatibility complex (MHC) class I and II surface antigens, and the late DC maturation marker CD83. The chemokine receptors CCR5 and CCR7 were also modulated on VLP-stimulated DCs, indicating that DC could migrate following VLP exposure. Furthermore, VLPs also elicited DC secretion of the pro-inflammatory cytokines TNF- α , IL-8, IL-6, and MIP-1 α . Most significantly, in stark contrast to DC treated with intact EBOV or MARV, DC stimulated with EBOV or MARV VLPs showed enhanced ability to support human T-cell proliferation in an allogenic mixed lymphocyte response (MLR). Thus, our findings suggest that unlike EBOV and MARV, VLPs are effective stimulators of DCs and have potential in enhancing innate and adaptive immune responses. © 2004 Elsevier Inc. All rights reserved.

Keywords: Filovirus; Virus-like particles; Dendritic cell; T cells

Introduction

The filoviruses, Ebola (EBOV) and Marburg (MARV), are nonsegmented, negative-strand RNA viruses that belong to the family *Filoviridae*. Filoviruses cause severe and often fatal hemorrhagic fever in humans and nonhuman primates (Lee and Henderson, 2001; Thacker, 2003; Whitfield, 2003). Natural outbreaks have been limited, but are currently on the rise (<http://www.who.int/disease-outbreaks-news/disease/bydisease.htm>). In sizable outbreaks, human mortality rates have approached 90% for EBOV and 70% for MARV (Bertherat et al., 1999; Feldmann and Klenk, 1996). High

mortality rates, significant transmissibility of disease, and a lack of therapeutic and preventative measures make filoviruses a serious threat to public health. At this time, there are no approved vaccines or therapeutics for treating or preventing EBOV and MARV infections. Hence, there is significant concern that these highly pathogenic viruses may be used as agents of mass casualty or bioterrorism.

The filovirus genome is 19 kb long, consisting of seven structural proteins and one nonstructural protein (Sanchez et al., 2001). Four of the viral proteins nucleoprotein (NP), viral proteins VP35, VP30, and the RNA-dependent RNA polymerase (L) are necessary for replication and transcription of viral RNA (Muhlberger et al., 1999), although glycoprotein (GP), VP40, and VP24 encode for membrane-associated virion proteins (Sanchez et al., 2001). The additional nonstructural EBOV protein is a secreted truncated form of the envelope glycoprotein (sGP) generated by reading of unedited mRNA (Feldmann and Klenk, 1996).

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GP is thought to be the only viral protein on the virion surface and it appears to be the principal, but probably not the only, mediator of protective immune responses (Hevey et al., 1998; Wilson et al., 2000). One of the obstacles in the development of preventive and therapeutic strategies against filoviruses is our limited understanding of the function of these proteins and the molecular details of the interaction of filoviruses and infected host cells.

The key for development of effective adaptive immunity to pathogens is rapid detection of a microbial presence and the subsequent activation of the host innate immune response. In recent years, dendritic cells (DCs) have been identified as a central cell for both activation of innate immune responses and initiation of adaptive immunity. DCs initiate immunity via a variety of pathways including secretion of a broad array of immunoregulatory molecules, presentation of antigen to B and T lymphocytes, interactions with natural killer cells, and direct cytotoxic activity against target cells (Lipscomb and Masten, 2002; Liu, 2001; Steinman, 2001). Following exposure to a pathogen or microbial-derived product, DCs undergo maturation and activation, which is indicated by phenotypic changes in specific cell-surface markers and secretion of large amounts of cytokines and chemokines (Liu, 2001). This maturation and activation process is vital, as it serves to make DCs one of the most efficient antigen-presenting cells in the body (Steinman, 2001).

We and others have shown that EBOV and MARV readily infect DCs and replicate logarithmically (Bosio et al., 2003; Geisbert et al., 2003; Mahanty et al., 2003). However, despite this impressive invasion and replication, filovirus-infected DCs fail to undergo phenotypic or functional maturation, as indicated by the absence of changes in cell-surface markers, lack of cytokine secretion, and interference with a mixed lymphocyte response (Bosio et al., 2003; Mahanty et al., 2003). Filovirus-infected DC likely do not appropriately initiate NK, B, and T-cell responses following EBOV or MARV infection *in vivo*, resulting in uncontrolled and disseminated infection of the host. At this time, the mechanism by which EBOV and MARV efficiently replicate in DC in the absence of stimulating immune responses is not understood. However, the interaction between DC and filoviruses may be a target for development of therapeutics for treating these devastating infections.

Recently, our laboratory reported that expression of both filovirus glycoprotein (GP) and the VP40 matrix protein leads to self-assembly of the viral proteins into filamentous virus-like particles (VLPs) with morphological similarity to filoviruses, but do not contain the viral genes necessary for replication (Bavari et al., 2002; Swenson et al., 2004). Importantly, the filovirus-like particles retain a morphology and antigenicity that is indistinguishable from native virus (Bavari et al., 2002; Swenson et al., 2004; Warfield et al., 2003). These two properties make VLPs attractive candidates for vaccine use, as well as tools for the study of virus/immune cell interactions. On the basis of previous observations with VLPs (Lenz et al., 2001; Marais et al., 1999;

Murata et al., 2003; Warfield et al., 2003), we hypothesize that unlike authentic EBOV and MARV, Ebola (e) and Marburg (m) VLP activate both innate and adaptive immunity. The objective of our current study was to determine the effects of eVLP and mVLP on human DCs. In this report, we show that filovirus VLPs are taken up by DCs and stimulate efficient maturation of human myeloid DCs, as indicated by changes in the cell-surface expression of CD40, CD80, CD83, CD86, MHC I, MHC II, CCR5, and CCR7 and by secretion of specific chemokines and pro-inflammatory cytokines including interleukin (IL)-6, IL-8, macrophage inflammatory protein (MIP)-1 α , and tumor necrosis factor (TNF)- α . Therefore, unlike EBOV and MARV, filovirus VLPs composed solely of GP and VP40 stimulates DCs. Together with our previous findings, these data indicate that activation and maturation of DCs by filovirus VLPs result in generation of both innate and adaptive immune responses.

Results and discussion

Ectopic expression of two filovirus proteins, VP40 and GP, resulted in formation of VLPs morphologically indistinguishable from EBOV and MARV (Bavari et al., 2002; Swenson et al., 2004). Additionally, we recently showed that injection of eVLP or mVLP completely protects rodents from homologous filovirus infection (Warfield et al., 2004, *in press*). The mechanisms of protection by the VLPs are unknown at this point in time. Development of a successful vaccine in higher primates is dependent upon knowledge of the requirements for protective immunity. It has been suggested that previous attempts to develop filovirus vaccines have failed in higher primates precisely due to this lack of understanding (Hart, 2003). Hence, our primary goal is to identify how the VLPs initiate immune responses and how this immunogenicity in specific cell types might aid in promoting lasting adaptive immune responses against EBOV and MARV. Antigen-presenting cells (APCs) are among the first immune cells to respond to viruses. Among APCs, DCs are the most efficient at initiating antigen-specific immune responses (Steinman, 2001). In their immature state, DCs reside in peripheral tissues where they continuously sample the environment and capture incoming pathogens. To determine if DCs were capable of internalizing VLPs, immature DCs were incubated with DiIC16-labeled VLPs and the cells examined by confocal microscopy for fluorescent VLPs within DCs. Both eVLP- and mVLP-labeled particles were found inside DCs within 2 h (Fig. 1). Examination of successive optical planes (taken at 0.5 μ m intervals) confirmed that VLPs were within the cells and not merely attached to the surface. To confirm that the particles visualized within the DCs were indeed VLPs and not extraneous cellular debris, VLPs were depleted from preparations by a combination of antibodies against Ebola or Marburg glycoprotein (GP). Following this depletion, we

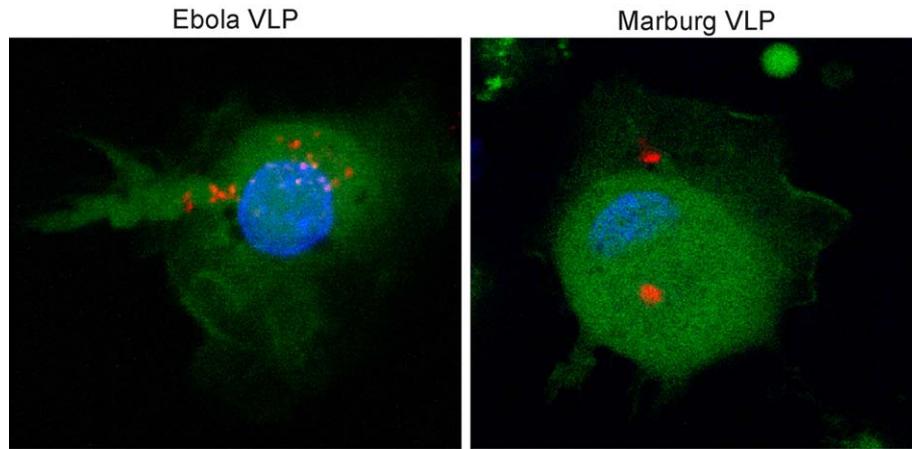


Fig. 1. Uptake of Ebola and Marburg VLPs by human DCs. DCs were stained with CellTracker Green and Hoescht nuclear stain. VLPs were stained with DiIC16 red dye. DCs were incubated for 2 h with the VLPs and unbound VLPs were washed away. VLP internalization was visualized by confocal microscopy as described in Materials and methods. VLPs are illustrated as large aggregates inside DCs. These images represent five experiments of similar design and outcome.

were unable to detect any particles within the DCs (data not shown), confirming that the DiIC16-labeled particles were indeed VLPs.

Uptake of foreign particles by DCs is not sufficient for inducing their maturation and subsequent contribution toward immunity against a pathogen. Maximal stimulation requires specific recognition and interaction between the pathogen and the DC (Lanzavecchia, 1999). Human myeloid DCs are readily infected by EBOV and MARV and infected DCs produce large titers of virus shortly following infection (Bosio et al., 2003; Mahanty et al.,

2003). However, DCs infected with filoviruses show little sign of sensing the viruses and infected DCs do not become mature or activated (Bosio et al., 2003; Mahanty et al., 2003). In striking contrast to live filoviruses, exposure of DCs to filovirus-like particles induced DC maturation (Fig. 2). Following VLP exposure, DCs increased expression of cell-surface costimulatory molecules including those essential for optimal activation of T cells: CD40, CD80 (B7.1), CD83, and CD86 (B7.2). Cell-surface receptors important for antigen presentation, such as major histocompatibility class I (HLA-ABC) and class

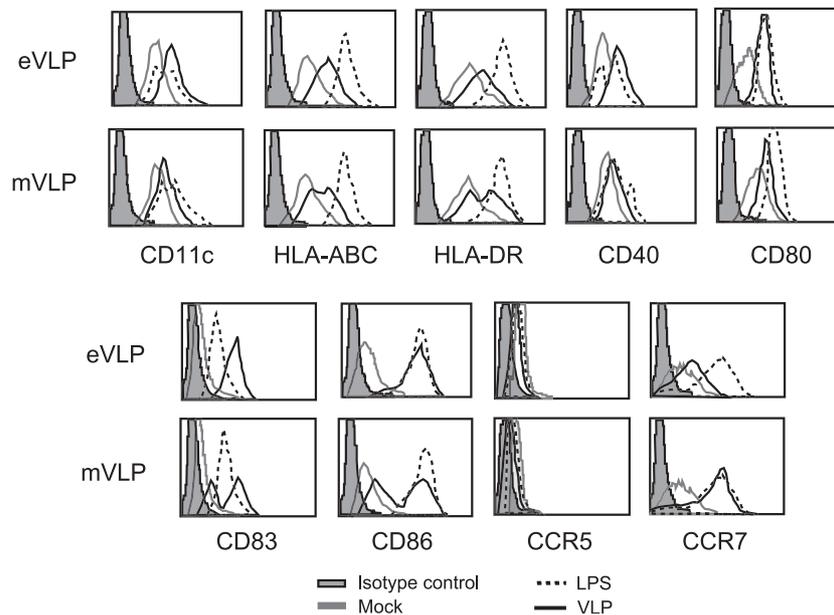


Fig. 2. Effect of VLP-induced phenotypic maturation of DCs and modulate chemokine receptors CCR5 and CCR7. DCs (10^6 cells/ml) were incubated with 10 μ g of eVLPs or mVLPs (solid black line). Controls were DCs treated with 10 ng LPS (Dashed line), or medium alone (gray line). Filled histograms represent staining with an appropriate isotype-matched, control antibody. The cells were harvested at 24 h post-inoculation and expression of CD80 (B7.1), CD86 (B7.1), CD40, CD11c, HLA-DR, HLA-ABC, and CD83 were analyzed by flow cytometry. Data are representative of three experiments of similar design and results.

II (HLA-DR) antigens, were upregulated on VLP-exposed DCs, as well as the cell adhesion molecule CD11c. Phenotypic maturation of DCs pulsed with VLPs was similar to that observed in DCs incubated in the presence of lipopolysaccharide (LPS), a known and potent stimulator of DC maturation *in vivo* and *in vitro* (Fig. 2).

As an additional measure of phenotypic DC maturation, it is critical to assess changes in specific chemokine receptors present on DCs. During the DC maturation process, there is small but measurable downregulation of the chemokine receptor CCR5, combined with the more obvious increased expression of CCR7. This coordinate regulation of chemokine receptors is presumed to enhance their ability to migrate from the afflicted tissue to resident lymph nodes (Jarrossay et al., 2001). Sustained expression of CCR7 allows DCs to perform their stepwise migration from tissue to the regional lymph node. The essential role of CCR7 in DC homing to lymphoid organs is supported by the observation that in CCR7-deficient mice, maturing DC are not able to migrate to lymph nodes (Förster et al., 1999). Thus, changes in these two chemokine receptors, in addition to other cell-surface markers discussed above, is essential for optimal DC function in the ensuing immune response. In support of the VLPs' ability to induce phenotypic maturation, we observed decreased surface expression of CCR5 and increased surface expression of CCR7 (Fig. 2). Interestingly, mVLP induced greater expression of CCR7 than eVLP 24 h poststimulation and

sustained greater CCR7 expression than LPS-stimulated and eVLP-stimulated DCs 48 h after exposure, although inactivated virus preparation failed to induce DC modulation of CCR5 or CCR7 (data not shown). Although downregulation of chemokine receptor CCR5 expression on DCs was observed, the differences in CCR5 expression among mock-, LPS-, VLP-stimulated, and inactivated virus were not remarkable.

In addition to phenotypic maturation, DCs should also undergo functional maturation to stimulate optimal innate and adaptive immune responses (Liu, 2001). To assess functional maturation, supernatants from VLP-treated DCs were analyzed overtime for a panel of cytokines and chemokines essential for potentiation of effective immune responses. In contrast to responses previously observed with infectious filoviruses, Ebola and Marburg VLPs induced secretion of IL-6, IL-8, MIP-1 α , and TNF- α (Fig. 3). To confirm that the secretion of cytokines from DCs was a specific result of VLPs interacting with DCs, preparations were depleted of VLPs using antibodies against EBOV or MARV GP. DCs incubated with these depleted preparations did not secrete cytokines or upregulate cell-surface markers (data not shown). As a control for nonspecific activation of the DC by any particle, we used inactivated filoviruses or inert latex beads. In contrast to the VLPs, neither inactivated EBOV or MARV nor latex beads elicited DC secretion of any of the cytokines we tested (unpublished data).

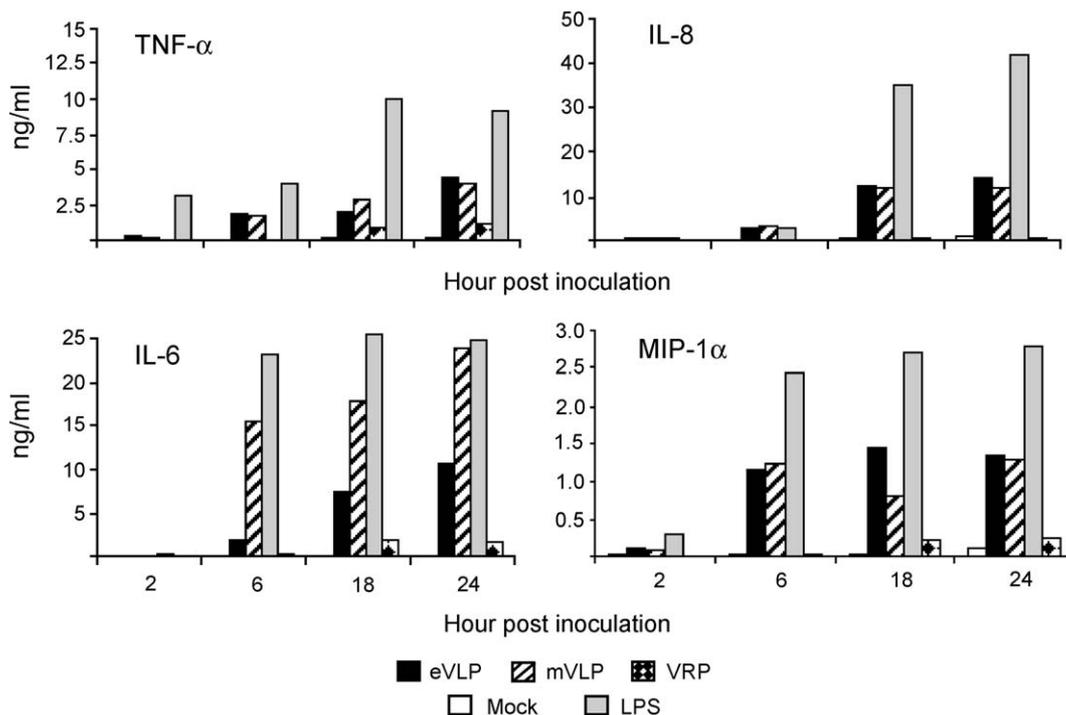


Fig. 3. Ebola and Marburg VLPs induce secretion of pro-inflammatory cytokines and chemotactic cytokines from human DCs. DCs (10^6 cells/ml) were incubated with $10 \mu\text{g}$ eVLPs or mVLPs, replication-deficient Venezuelan equine encephalitis replicons (VRP) encoding EBOV GP (m.o.i. of 1.0), 10 ng of LPS, or medium only (mock). Concentration of each cytokine in duplicate culture supernatants was measured at 2, 6, 18, and 24 h after addition of stimulant as described in the Materials and methods. These results are representative of three experiments of similar design and results.

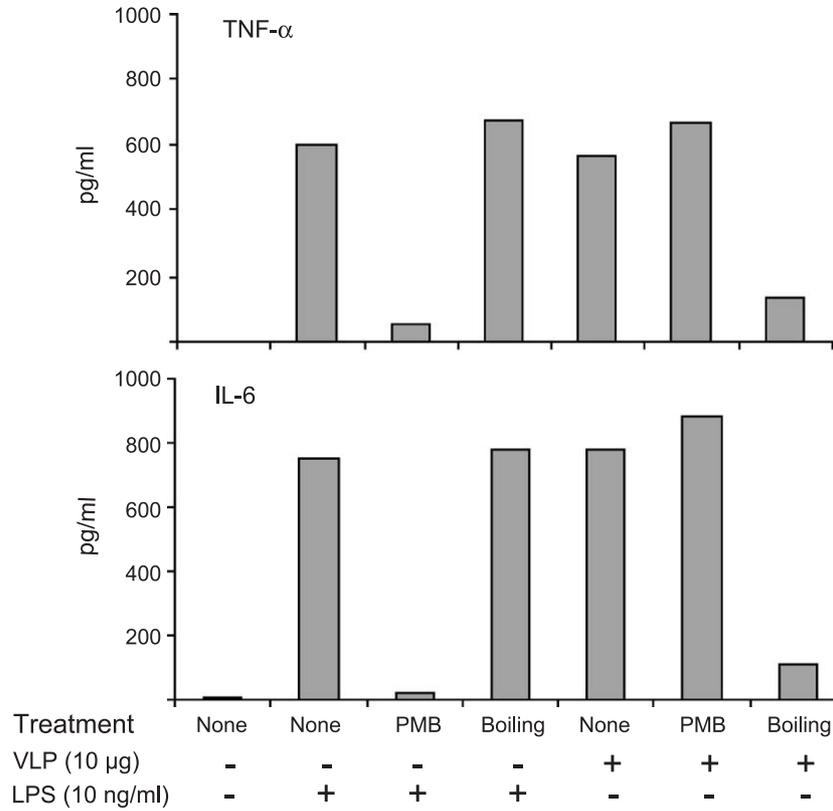


Fig. 4. VLP protein structure, not LPS contamination, is responsible for the functional responses of DCs to eVLPs. To determine whether LPS contamination was responsible for the DC cytokine secretion following VLP exposure, 10 μg/ml polymyxin B was incubated eVLPs or LPS at room temperature. To determine whether the protein structure of the eVLPs were responsible for the biological responses of the DCs to VLPs, either VLPs or LPS were subjected to boiling at 100 °C. Equivalent amounts of VLPs and LPS were mock-treated. Following 1 h of the indicated treatment, 10 μg/ml of VLPs or 10 ng/ml of LPS were added to DCs, as above. The DC supernatants were removed after 18 h and analyzed by CBA for IL-6 and TNF-α. These results are representative of two experiments of similar design and outcome.

Because secretion of many of the cytokines that were examined here could be also potentiated by endotoxin, we added polymyxin B, a compound that binds and inactivates

LPS biological functions (Jacobs and Morrison, 1977) to the VLPs and reexamined DC responses to polymyxin B-treated VLPs. We observed no alteration in the cytokine or chemo-

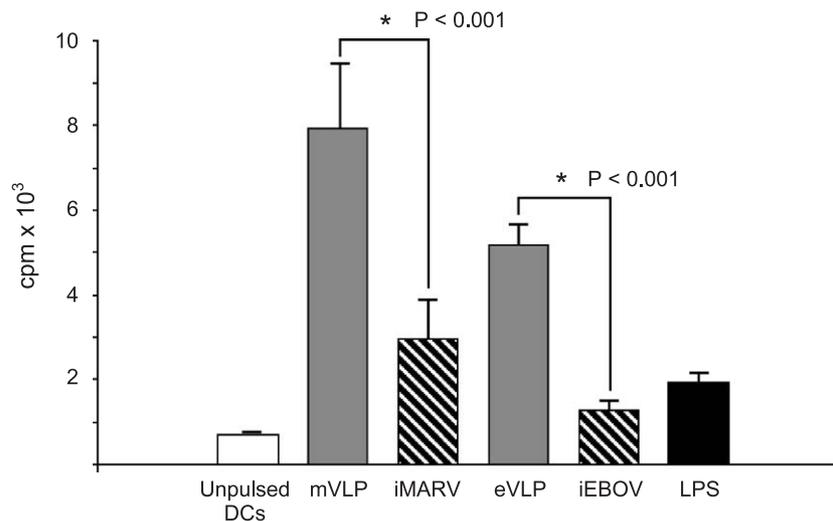


Fig. 5. Marburg and Ebola VLP enhance allogenic T-cell stimulation. One million DCs were stimulated with 10 μg eVLP or mVLP, 50 μg of inactivated Ebola or Marburg virus, 10 ng of LPS, or mock-treated for 24 h. Subsequently, DCs were washed and cocultured at graded numbers with purified allogenic T cells (2.5 × 10⁵ per/well) for 4 days. T-cell proliferation was assayed by [³H]thymidine incorporation. The results are expressed as mean CPM ± SD of two independent experiments. Statistically significant differences (P ≤ 0.05) were determined between the VLP-treated and inactivated virus-treated groups.

kine response upon addition of polymyxin B to VLPs, although addition of polymyxin B to LPS inhibited cytokine production by DC (Fig. 4). Furthermore, boiling the VLPs, which destroys VLP protein structure, significantly inhibited the cytokine-inducing activity of the VLP. As expected, boiling of LPS had no effect on the cytokine-inducing activity of the VLP-stimulated DCs. Hence, the protein structure of the VLPs, and not LPS contamination, are the cause of the biological responses seen in VLP-exposed DCs. Together, these data indicated that the filovirus-like particles, in contrast to live filoviruses, specifically interacted with human dendritic cells to induce their maturation and activation.

Mature DCs are the most efficient cell for induction of activation, differentiation, and proliferation of unprimed T cells. EBOV and MARV interfere with DC-mediated T-cell proliferation; thus, we investigated the effect of VLP-pulsed DCs on T-cell proliferation in an allogenic mixed lymphocyte response (MLR). DCs were incubated for 24 h with eVLP, mVLP, inactivated EBOV (iEBOV), or inactivated MARV (iMARV). Allogenic T cells were then added to treated DCs. Unlike iEBOV or iMARV, DCs pulsed with eVLP or mVLP induced significant T-cell proliferation (Fig. 5; $P < 0.001$). Therefore, although native EBOV and MARV did not support the ability of DC to induce proliferation of T cells, VLP-stimulated DCs efficiently promoted primary T-cell proliferative responses, likely due to the upregulation of costimulatory and major histocompatibility molecules on the activated DCs (Fig. 2).

EBOV, live or inactivated, interfere with or avoid DC responses (Figs. 2 and 3; Bosio et al., 2003; Mahanty et al., 2003). The specific protein(s) responsible for this interference have not been fully identified. In the study presented here, we have attempted to identify how VLPs might successfully modulate DC immune responses. Although EBOV GP has been proposed to modulate host adaptive immune responses (Feldmann et al., 1999), we have comprehensively demonstrated that eVLP, which consists only of GP and VP40, activate DCs. This suggests that GP presented in conjunction with VP40 in particle form probably is not responsible for downregulation or interference of DC responses to EBOV. Based on the data that are presented here, it seems that VP40 is also not responsible for down modulation of the DC response to filovirus infection. When expressed alone, VP40 is spontaneously released from cells (Jasenosky et al., 2001; Timmins et al., 2001). However, studies within our laboratory show that the release of filamentous structures by mere expression of VP40 was far less efficient compared with cells transfected with both GP and VP40 (unpublished data). Furthermore, the matrix protein VP40 is not thought to be exposed on the virus surface, and VP40 particle structures lack the typical spikes of the filoviruses (Jasenosky et al., 2001; Timmins et al., 2001). The importance of immune responses to VP40 are questionable because administration of VP40 alone cannot completely protect mice from lethal EBOV challenge, nor

guinea pigs from lethal MARV challenge (unpublished data and Hevey et al., 1998; Wilson et al., 2001). Another candidate for modulation of the DC responses following filovirus infection is the IFN antagonist VP35. EBOV VP35 can block the phosphorylation and dimerization of the transcription factor interferon regulatory factor 3, which prevents activation of critical antiviral genes (Basler et al., 2000, 2003; Bosio et al., 2003). Although the specific mechanisms are unclear at this time, the virulence of EBOV and MARV likely depends on their ability to evade or downregulate the innate immune cell responses to viral infections, especially early responders such as DCs. VLPs with additional structural proteins, such as VP35, incorporated may allow us to dissect the immunomodulatory role of individual virion components.

Previously, we reported that eVLP induced maturation and activation of mouse bone marrow-derived DCs. Injection of eVLP activated both B and T lymphocytes within 3 days and eVLP vaccination of mice resulted in complete protection against lethal EBOV challenge (Warfield et al., 2003). Similarly, mVLP vaccination of guinea pigs induced MARV-specific antibody and T-cell responses that protected from lethal MARV infection (Warfield et al., 2004, in press). In this report we utilized filovirus VLP as a tool to identify the immunogenic nature of particles consisting of GP and VP40 in human DCs. We showed activation and maturation of human myeloid DCs by filovirus VLPs, which can result in stimulation of primary T-cell responses. This work supports further testing of VLP as vaccine candidates and suggests immunogenicity in humans. Beyond their application as vaccines, the immunogenicity of Ebola and Marburg VLP in human DC described herein also points toward their usefulness and informative application in the understanding of filovirus interaction with multiple components of the host immune response. These types of efforts should ultimately lead to better understanding of filovirus pathogenesis.

Materials and methods

Generation of Ebola and Marburg virus-like particles

Filovirus VLPs were generated as previously described (Bavari et al., 2002). Briefly, cDNAs encoding Ebola virus strain Zaire 1995 (EBOV) or Marburg virus strain Musoke (MARV) GP and the corresponding matrix protein VP40 were cloned into the mammalian expression vector pWRG7077. 293T cells were transfected using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen, Carlsbad, CA). VLPs were collected from cell-free supernatants by an initial high-speed centrifugation followed by purification on sucrose gradients. Confirmation of the presence of VLPs and their quality was performed using protein quantification, electron microscopy, Western

blotting, and silver staining following gel electrophoresis, as previously described (Bavari et al., 2002; Swenson et al., 2004; Warfield et al., 2004, in press) All VLP preparations were tested for endotoxin using a LAL assay (BioWhittaker, Walkersville, MD) and were found to be <0.03 IU endotoxin/ μg total protein.

Culture of primary human DCs

Primary adherent human monocytes were isolated following enrichment of peripheral blood monocytes by centrifugation over ficoll-hypaque and resuspended in complete (c) RPMI-1640 (Life Technologies, Rockville, MD) supplemented with 2 mM L-glutamine (Life Technologies, Grand Island, NY), 10 mM HEPES buffer (Life Technologies), and 1% nonessential amino acids (NEAA, Life Technologies). Cells were allowed to adhere (2 h at 37 °C) after which nonadherent cells were removed. The adherent cells were cultured in cRPMI-1640 supplemented with 100 ng/ml of recombinant human GM-CSF (rhGM-CSF) and 20 ng/ml of recombinant human IL-4 (rhIL-4) (both from R&D Systems, Minneapolis, MN). One-third of the medium and 100% of each cytokine were replaced every other day until use. The resulting differentiated DC were $>97\%$ CD1a positive and $<1\%$ CD14 negative. Immature DCs were used on the fourth day of culture.

Staining of VLPs and confocal imaging

VLPs were incubated with the lipophilic dye DiIC16 (Molecular Probes, Eugene, OR) diluted 1:5000 in PBS for 10 min at room temperature. Unincorporated dye was removed following addition of PBS and centrifugation. Before addition of stained VLPs, DCs were incubated with CellTracker Green CMFDA (Molecular Probes) for 1 h in which the last 10 min Hoescht nuclear stain (Molecular Probes) was also added. DCs were incubated with VLPs at 37 °C/5% CO₂ for 2 h before visualization using a Radiance 2000 MP multiphoton confocal microscope with LaserSharp 2000 Software (Bio-Rad, Hercules, CA).

Stimulation of DCs

DCs (1×10^6 cells) were incubated with 10 μg Ebola or Marburg VLP, 50 μg of irradiated (1.0×10^7 rad) EBOV or MARV, 10 ng/ml lipopolysaccharide (LPS, Sigma, St. Louis, MO), or replication-deficient Venezuelan equine encephalitis replicons encoding EBOV GP (VRP) as previously described (Pushko et al., 2000). Briefly, immature human DCs were incubated with VRP, VLP, VLP preparations depleted of VLPs using anti-GP antibodies, LPS, or mock-infected at 5×10^5 DCs per well in 24-well plates. In some assays, 10 $\mu\text{g}/\text{ml}$ polymyxin B was incubated with 10 $\mu\text{g}/\text{ml}$ of VLPs or 10 ng/ml LPS at room temperature for 1 h. Alternately, equivalent amounts of VLPs and LPS were mock-treated or were subjected to boiling at 100 °C for 1 h.

Following treatment, the VLPs or LPS were added to DCs, as above. At designated time points, supernatants were removed and frozen at -70 °C until analysis of cytokines could be performed. At the designated time points, DCs were stained for specific cell-surface markers and analyzed by flow cytometry as described below.

Assay of cytokines in DC culture supernatants

Culture supernatants were assayed for MIP-1 α using Biosource kits (Camarillo, CA) or TNF- α , IL-6, and IL-8 using cytometric bead array (Pharmingen, San Diego, CA). Assays and controls conformed to the manufacturer's instructions.

Flow cytometry

Dendritic cell-surface markers were analyzed by flow cytometry as previously described (Sallusto and Lanzavecchia, 1994). Briefly, cells were washed in cold PBS supplemented with 2% fetal bovine serum and cell-surface staining was done using the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CCR5, FITC-conjugated anti-CCR7, phycoerythrin (PE)-conjugated anti-CD83, FITC-conjugated anti-CD80, PE-conjugated anti-CD86, FITC-conjugated anti-HLA-DR, PE-conjugated anti HLA-ABC, FITC-conjugated anti-CD40, PE-conjugated anti-CD11c, FITC-conjugated anti-mouse IgG₁^k, and PE conjugated anti-mouse IgG2_b^k (all from Pharmingen). After incubation with antibodies, cells were washed and fixed with 10% buffered formalin and analyzed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) with CellQuest software (Becton Dickinson). A minimum of 10,000 events were collected and analyzed for each sample.

Allogenic T-cell proliferation

Human T cells were enriched from peripheral blood mononuclear cells (PBMC) by negative selection using Dynal T-cell negative isolation kit (Dynal Biotech, Norway). Allogenic DCs stimulator cells were pulsed with 10 $\mu\text{g}/\text{ml}$ of VLPs, inactivated Ebola or Marburg virus (m.o.i. of 10), LPS, or mock-infected for 24 h. DCs (5×10^3 /well) were subsequently washed, resuspended in cRPMI that contained heat-inactivated human AB serum (BioWhittaker), and added at graded doses to purified responder T cells (2.5×10^5 /well) in 96-well round-bottom microtiter plates (Corning, Inc., Corning, NY) in a total volume of 200 μl . Triplicate cultures were maintained at 37 °C/5% CO₂ for 5 days, after which time T-cell proliferation was measured by incorporation of [³H]thymidine ([³H]TdR) (Amersham, Arlington Heights, IL). Each well received 1 μCi [³H]TdR and was subsequently harvested 18 h later. Results are represented as mean CPM \pm SD of triplicate wells.

Data analysis

Data were analyzed using a paired Student's *t* test, and results were considered significant if $P \leq 0.05$.

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