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Role of Natural Killer Cells in Innate Protection against Lethal Ebola Virus Infection

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Abstract

Ebola virus is a highly lethal human pathogen and is rapidly driving many wild primate populations toward extinction. Several lines of evidence suggest that innate, nonspecific host factors are potentially critical for survival after Ebola virus infection. Here, we show that nonreplicating Ebola virus-like particles (VLPs), containing the glycoprotein (GP) and matrix protein virus protein (VP)40, administered 1–3 d before Ebola virus infection rapidly induced protective immunity. VLP injection enhanced the numbers of natural killer (NK) cells in lymphoid tissues. In contrast to live Ebola virus, VLP treatment of NK cells enhanced cytokine secretion and cytolytic activity against NK-sensitive targets. Unlike wild-type mice, treatment of NK-deficient or -depleted mice with VLPs had no protective effect against Ebola virus infection and NK cells treated with VLPs protected against Ebola virus infection when adoptively transferred to naive mice. The mechanism of NK cell–mediated protection clearly depended on perforin, but not interferon-γ secretion. Particles containing only VP40 were sufficient to induce NK cell responses and provide protection from infection in the absence of the viral GP. These findings revealed a decisive role for NK cells during lethal Ebola virus infection. This work should open new doors for better understanding of Ebola virus pathogenesis and direct the development of immunotherapeutics, which target the innate immune system, for treatment of Ebola virus infection.

Key words: virus-like particles • filoviruses • immunity • matrix protein • glycoprotein

Introduction

The filovirus Ebola (Ebola virus) quickly outpaces the innate immune response of the host and causes an acute, progressive hemorrhagic fever with mortality rates of up to 90% (1, 2). The key initiators of innate immunity, including monocytes, macrophages, and DCs, appear to be the primary targets of filovirus infection (3–6). Ebola virus replicates efficiently in DCs without eliciting cytokine and chemokine secretion, and infected DCs fail to mature and alert other critical mediators of early and adaptive immune responses (5, 6). This lack of DC activity most likely results in poor immune responses by NK, T, and B cells, which in turn contribute to the uncontrolled spread and growth of the virus. In contrast, the early initiation of innate proinflammatory responses correlates with the survival of Ebola virus–infected humans (7–10). Therefore, the rapid initiation of early immune responses may limit Ebola virus infection, and is critically linked to host survival.

NK cells are key components of the innate immune system, rapidly responding to invading microbes by exocytosis of perforin and granzymes, which mediate the destruction of infected cells (11). Additionally, NK cell secretion of cytokines such as IFN-γ, IFN-α/β, and TNF-α serve a dual purpose in that they initiate the immediate activation of antimicrobial pathways in infected cells, followed by modulation of adaptive responses to the pathogen (11–13). The

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Abbreviations used in this paper: GP, glycoprotein; MCMV, murine cytomegalovirus; MIP, macrophage inflammatory protein; moi, multiplicity of infection; VLP, virus-like particle; VP, viral protein.

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induction of cytokines and chemokines by viral infections is also known to trigger NK cell activity. Specifically, virus-induced IFN-α/β enhances NK cell–mediated cytotoxicity. Alternately, the induction of IL-12 by some viral infections is responsible for the production of high levels of IFN-γ by NK cells, as well as the induction of NK cytotoxic activity (11).

NK cells appear to play a critical role in the immune response to Epstein-Barr virus, murine cytomegalovirus (MCMV), and herpes simplex virus-1 (14–16). The clinical importance of NK cells to antiviral immunity is documented by the fact that recurrent herpesvirus infections have been observed in a NK-deficient patient (17). NK cell activity is closely regulated by a myriad of activating and inhibiting cell surface receptors, and consequently, viruses have evolved multiple mechanisms to evade or modulate these receptors. Such mechanisms include the up-regulation of HLA-C and HLA-E molecules on the surface of virus-infected cells, expression of viral MHC homologues to trigger NK inhibitory receptors, and/or the release of cytokine homologues with inhibitory activities (11, 12, 14). In contrast, virus-infected cells often down-regulate MHC class I on their surface, which enhances NK cell–mediated lysis due to removal of the inhibitory signals delivered by the MHC.

There is a limited understanding of the interactions of the innate immune response and, in particular, NK cell responses to Ebola virus infection. To investigate the role of early, innate immune responses to Ebola virus, we established a model in the context of the viral proteins, presented as virus-like particles (VLPs). We have shown previously that VLPs, comprised of the Ebola virus glycoprotein (GP) and matrix protein virus protein (VP)40, efficiently mature and activate murine myeloid DCs (18). In addition to their potent activation of DCs, which are critical mediators of innate and adaptive immune responses, VLPs activate T and B cells in vivo after intraperitoneal administration to mice (18). VLPs are highly immunogenic in mice in the absence of adjuvant and provide a much-needed tool for safely dissecting immune responses to filoviruses. Therefore, during these investigations, we used the genome-free Ebola VLPs to study the contribution of NK cells to innate immune responses to lethal Ebola virus infection.

Materials and Methods

Virus and Cell Lines. The wild-type strain of Ebola virus–Zaire was originally isolated from a fatally infected human in 1995 (19). The Ebola virus–mouse-adapted strain was generated by serial passage in progressively older mice (20). Ebola virus was propagated and viral titers were assessed by standard plaque assay in Vero E6 cells (19, 20). Inactivated Ebola virus–Zaire 1995 preparations were purified from cell-free supernatants on continuous sucrose gradients and irradiated with 10^7 rad, as described previously (21). All experiments with Ebola virus were performed under maximum containment in a biosafety level-4 laboratory at the United States Army Medical Research Institute of Infectious Diseases.

Mice. BALB/c, C57Bl/6, and IFN-γ–deficient (C57Bl/6 background) mice were obtained from the National Cancer Institute. Perforin-deficient (BALB/c background) mice were provided by T. Sayers (National Cancer Institute, Frederick, MD). NK cell–deficient mice were generated and bred at Washington University (22). NK cells were depleted from C57Bl/6 mice by intraperitoneal injection of 50 μl of anti-asialoGM antibodies (Wako Chemicals USA, Inc.) every other day from −5 to +5 d after challenge. Control mice were treated in the same manner using rabbit Ig (Sigma-Aldrich). Mice (6–12-wk-old) were divided randomly into experimental groups, housed in microisolator cages, and provided food and water ad libitum. 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. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

VLP Preparation. To generate VLPs, 293T cells were cotransfected with pWRG vectors encoding for Ebola virus VP40 and GP (VLP) or Ebola virus VP40 alone (VLP_{VP40}) using Lipofectamine 2000 (Invitrogen). To purify the VLPs, the cell-free supernatants were harvested after 2–3 d and pelleted at 9,500 *g* for 4 h. These crude preparations were separated on a 20–60% continuous sucrose gradient by ultracentrifugation overnight. The gradient fractions were concentrated by a second centrifugation, resuspended in endotoxin-free PBS, and the fractions containing the VLPs were determined using Western blots and electron microscopy. As a control, cell-free supernatants from 293T cells transfected with an empty pWRG vector were purified in an exact manner as the VLP preparations. Only a very small amount of cell-free supernatants from mock-transfected cells could be generated, and experiments with these sucrose-purified supernatants resulted in a similar outcome to medium alone. Therefore, the sucrose-purified cell-free supernatants were only used in select experiments. The amount of inactivated Ebola virus and VLP in each preparation was quantitated using a semi-quantitative Western blot for VP40 along with a measurement of total protein concentration, obtained by disrupting the samples with NP-40 detergent before use in a detergent-compatible protein assay (Bio-Rad Laboratories). The VLP preparations used in this work were <0.03 U/mg endotoxin as determined by the *Limulus* amebocyte lysate test (Biowhittaker).

VLP Injection and Ebola Virus Challenge of Mice. For protection experiments, mice were injected intraperitoneally or intramuscularly with 25 μg of VLP, VLP_{VP40}, inactivated Ebola virus, or PBS alone 1, 2, or 3 d before challenge with mouse-adapted Ebola virus. Mice were challenged by intraperitoneal injection. As noted, mice were injected with 10 or 100 PFU of mouse-adapted Ebola virus (>300 or >3,000 LD_{50}, respectively; reference 20). After challenge, mice were observed at least twice daily for illness and death for at least 28 d; no changes were observed in the health of any mice in these studies between 14 and 28 d after infection.

Flow Cytometry. The spleen or mediastinal lymph nodes were collected from individual mice and placed in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 1 mM Hepes, and 0.1 mM of nonessential amino acids (referred to as complete RPMI 1640). Single cell suspensions of lymphocytes were produced from each sample, the red blood cells were lysed with ACK lysis buffer, and the phenotypic expression of cells was examined by flow cytometry with NK1.1-FITC (BD Biosciences). Intracellular IFN-γ in NK cells was detected after fixation and permeabilization using Cytofix/Cytoperm™ (BD Biosciences), staining with PE-labeled IFN-γ, and analysis by flow cytometry

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as described aforementioned. The percent of positive events was determined after collecting 50,000 events (gated based on forward and side scatter for viable lymphocytes) per sample using CELL-Quest™ software on a FACSCalibur® (BD Biosciences).

Enrichment and Depletion of NK Cells. NK cells were isolated from the livers of mice after a hydrodynamic shearing method, which was used to increase the numbers of NK cells obtained from each mouse, unless noted otherwise (24, 25). In brief, mice received a hydrodynamic shear, or rapid tail vein injection, with 5 μg IL-2 plasmid in 1.6 ml of 0.9% normal saline. 3–4 d after the injection, lymphocytes were isolated using a 40–80% Percoll® step gradient from perfused livers of the IL-2–treated mice. The NK cell preparations were obtained by negative selection using biotinylated CD3, CD4, CD8, and CD19 antibodies (BD Biosciences) followed by streptavidin MicroBeads (Miltenyi Biotec). The NK preparations were routinely 85–95% pure based on flow cytometry analysis for cell surface expression of NK1.1, both before and after overnight stimulation. The NK cell–enriched preparations contained 3–10% eosinophils, based on forward and side scatter and CD11b expression, 1–3% $B220^+MHC$ class II^+ DCs, B cells, and 1–2% $CD5^+$ T cells and did not contain $CD3^+$ NK T cells (unpublished data). To deplete the NK cells from the NK cell–enriched preparations, the cells underwent a second negative selection using biotinylated NK1.1 antibodies (BD Biosciences) and streptavidin magnetic beads (yielded >90% NK cell depletion).

Cell Stimulations and Blocking Studies. NK cells (10^6 cells/ml of complete RPMI 1640) were stimulated for 2–72 h with 100 iU/ml of murine IL-2 (PeproTech), 10 μg/ml polyI:C, or 0.1–50 μg of inactivated Ebola virus, VLPs, or sucrose-purified cell-free supernatants from mock-transfected cells. To assess the role of LPS contamination on NK cell cytokine secretion, 50 μg/ml of VLPs or 10 ng/ml LPS was incubated for 1 h with 100 μg/ml of polymyxin B at room temperature (26) or boiled for 1 h before their addition to NK cell preparations. In the blocking experiments, 10 μg of VLPs were incubated with either a pool of three mAbs against Ebola virus GP (10 μg each; reference 27), 30 μg of an anti-Ebola virus VP40 mAb, 30 μl of mouse sera from mice vaccinated with either a replication-deficient Venezuelan equine encephalitis particle vaccine (VRP) expressing Ebola VP40 or Lassa N (a gift of M.K. Hart, United States Army Medical Research Institute of Infectious Diseases, Frederick, MD; reference 28), or 30 μg of anti–human CD2 antibody (BD Biosciences). Percent inhibition of IFN-γ secretion was calculated as follows: [IFN-γ secretion with test antibody/IFN-γ secretion with control antibody (hCD2)] × 100%.

Cytotoxicity Assay. A standard 4-h ^{51}Cr assay was used to assess the cytotoxic activity of the stimulated NK cells (29). Varying numbers of stimulated NK cells were added to 5,000 ^{51}Cr-labeled YAC-1 target cells for 4 h. The amount of ^{51}Cr released into the supernatants of each sample was determined and the specific lysis was assessed by: [(sample cpm-spontaneous release)/(total release-spontaneous release)] × 100%.

Cytokine Detection. Concentrations of IFN-γ and TNF-α present in culture supernatants were measured by cytometric bead array (BD Biosciences) per the manufacturer's directions. The concentration of IFN-γ, macrophage inflammatory protein (MIP)–1α, and TNF-α present in the Ebola virus–treated NK cell supernatants was tested by ELISA (R&D Systems) under biosafety level-4 containment.

NK Cell Transfers. After overnight stimulation, NK cells were washed twice and enumerated. 5 × 10^6 viable NK cells were resuspended in PBS and injected intraperitoneally into naive mice. The recipient mice were challenged 6 h later with Ebola virus, and illness and survival were scored for 28 d.

Statistical Analysis. A paired Student's *t* test was used to directly compare treated and mock-treated samples. The proportion of treated and control animals surviving was compared by one-tailed Fisher exact tests within experiments. For survival experiments with more than one treatment group, adjustments for multiple comparisons were made by stepdown Bonferroni correction. Analyses were conducted using SAS Version 8.2 (SAS Institute Inc.). A p-value of ≤0.05 was considered significant.

Results

VLPs Rapidly Induce Protection from Lethal Ebola Virus Infection. Morphologically, VLPs are almost indistinguishable from inactivated Ebola virus by electron microscopy (18, 23) or by atomic force microscopy (Fig. 1 A and reference 2). The VLPs induced potent innate immune re-

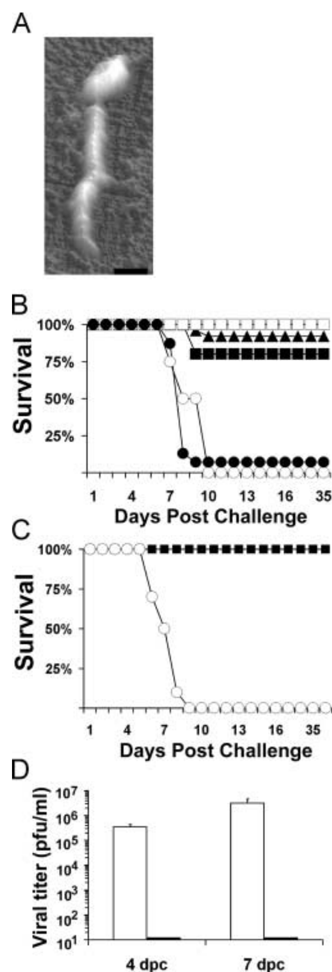

Figure 1. Ebola VLPs induce rapid protective responses against Ebola virus infection. (A) Atomic force micrograph of a VLP, courtesy of M. Thompson (Veeco Instruments, Woodbury, NY). Bar, 0.25 μm. (B) C57Bl/6 mice were primed intraperitoneally with 25 μg of VLPs (□) 1 d (n = 10), (▲) 2 d (n = 10), or (■) 3 d (n = 30) before challenge; (○) irradiated, inactivated Ebola virus (n = 10); (●) sucrose-purified supernatants from mock-transfected cells or PBS (n = 30) 3 d before challenge with 100 PFU of mouse-adapted Ebola virus. Results are plotted as percent survival for each group, and the survival curves were constructed using data from two to five separate experiments. Treatment with VLPs 1–3 d before challenge significantly increased the proportion of the mice surviving challenge ($P < 0.0001$) compared with mice treated with Ebola virus infection or sucrose-purified supernatants from mock-transfected cells, based on a one-way Fisher's exact test. (C) One intramuscular injection with (■) VLPs or (□) PBS was administered to C57Bl/6 mice (n = 10/group) 3 d before challenge with 100 PFU of mouse-adapted Ebola virus. Results are plotted as percent survival for each treatment group. The data were generated in two separate experiments with five mice per group. A significant increase in survival was observed in VLP-treated mice compared with PBS-treated mice ($P < 0.0001$). (D) One intraperitoneal injection of PBS (white bars) or VLP (black bars) was administered to C57Bl/6 mice 3 d before challenge with 100 PFU of mouse-adapted Ebola virus. Serum was collected from the VLP- or PBS-vaccinated mice 4 or 7 d after challenge with Ebola virus and assayed for viral titers by plaque assay. Data are represented as the mean ± SD (n = 5).

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sponses, as mice injected once intraperitoneally with VLPs, 1–3 d before challenge with >3,000 LD_{50} of Ebola virus (20) were 80–100% protected from death (Fig. 1 B). However, mice injected 3 d before challenge with either irradiated, inactivated Ebola virus or the sucrose-purified supernatants from mock-transfected cells succumbed to Ebola virus challenge (Fig. 1 B). Irradiating the VLPs had no effect on the outcome of these experiments (unpublished data), suggesting that the failure of the inactivated Ebola virus to protect mice from Ebola virus infection was not simply due to the irradiation. Intramuscular injection of VLPs also induced high levels of protection against Ebola virus challenge (Fig. 1 C), indicating that the route of VLP administration was not linked to protection from Ebola virus lethality. Circulating Ebola virus was undetectable at 4 or 7 d after Ebola virus challenge in VLP-treated mice, whereas control mice exhibited high circulating viral titers after Ebola virus infection (Fig. 1 D). Protection elicited within 1–3 d of VLP injection suggested that VLPs activated innate immune responses. Therefore, this approach gave us a vital tool to investigate early protective cellular responses to Ebola virus.

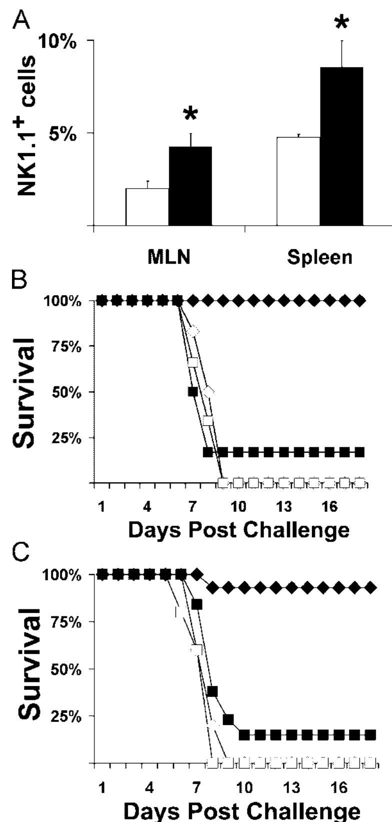

Figure 2. The innate protection against Ebola virus mediated by VLPs requires functional NK cells. (A) Mediastinal lymph node or splenic cells from mice injected with VLP (black) or PBS (white) were evaluated for cell surface expression of NK1.1 by flow cytometry. These data represent the mean of the number of $NK1.1^+$ cells in each organ ± SD. *, $P \leq 0.001$ for the VLP-injected mice compared with the control mice by Student's paired *t* test ($n = 5$). Similar results were obtained in two separate experiments. (B) NK cell–deficient mice ($n = 6$/group) were injected intraperitoneally with 25 μg of VLPs (■) or media (□). As controls, C57Bl/6 mice ($n = 6$/group) were administered VLPs (◆) or media (◇). 3 d later, the mice were challenged with 100 PFU of mouse-adapted Ebola virus. Results are plotted as percent survival for each group. A significant decrease in the survival of VLP-treated NK cell–deficient mice was observed, as compared with the VLP-treated C57Bl/6 control mice ($P = 0.0076$). (C) NK cells were depleted from C57Bl/6 mice by intraperitoneal injection of 50 μl of anti-asialoGM antibodies every other day from −5 to +5 d after challenge. Control mice were treated identically using rabbit Ig (Sigma-Aldrich). NK cell–depleted mice were injected intraperitoneally with 25 μg of VLPs (■, $n = 13$) or media (□, $n = 5$) 3 d before challenge or control-treated mice were administered VLPs (◆, $n = 5$) or media (◇, $n = 5$) 3 d before challenge. The mice were challenged with 100 PFU of mouse-adapted Ebola virus. Percent survival for each group is shown. A significant difference in the survival of VLP-treated NK cell–depleted mice was found when compared with the VLP-treated C57Bl/6 control mice ($P = 0.0001$).

Innate Protection against Ebola Virus Requires NK Cells. Although many different factors may have contributed to VLP-induced innate protection, we narrowed our search to the role of NK cells. Marked increases in NK cell activity occur early in microbial invasions and result in the recruitment of NK cells to the site of infection (29). VLPs recruited almost twice the number of NK cells in both the mediastinal lymph node and spleen compared with animals receiving PBS alone (Fig. 2 A), suggesting that VLP administration induces NK cell proliferation and/or trafficking in lymphoid tissues. To directly examine the role of NK cells in Ebola virus infections, NK cell–deficient mice (22) were administered VLPs 3 d before lethal Ebola virus challenge. VLP pretreatment of mice lacking functional NK cells did not protect from Ebola virus infection (Fig. 2 B, 1/6), unlike VLP-injected wild-type C57Bl/6 mice (6/6, $P = 0.0076$). Furthermore, mice depleted of NK cells using anti-asialoGM1 antibodies were not protected by VLP treatment (Fig. 2 C, 2/13 survivors), unlike VLP-treated C57Bl/6 mice (14/15 survivors, $P = 0.0001$). Although anti-asialo-GM1 antibodies can deplete both NK cells and cells of a monocytic lineage, together these data directly implicated NK cells in the rapid protection mediated by VLPs.

Because NK cells were required for protection against Ebola virus infection, we examined whether VLPs induced the functional activation of NK cells in vitro. To enhance the number of NK cells and to obtain highly enriched preparations of NK cells, we used a cDNA hydrodynamic shearing method (24, 25). After the rapid tail vein injection of IL-2 plasmid, a substantial increase was observed in the number of NK cells in the liver (unpublished data). To determine the effect of this procedure on NK cells, we obtained NK cells from livers of untreated or sheared C57Bl/6 mice and found no differences in cytokine profiles when these cells were stimulated with IL-2, VLPs, or sucrose-purified cell-free supernatants from mock-transfected cells (Fig. 3 A). The VLPs, but not inactivated Ebola virus, induced IFN-γ and TNF-α secretion from NK cells (Fig. 3, B and C). NK cells activated with VLPs also secreted IL-4, IL-5, IL-6, IL-13, and MIP-1α, but not detectable IL-2 and IFN-α (unpublished data). We performed intracellular staining for IFN-γ and surface staining for NK1.1 to con-

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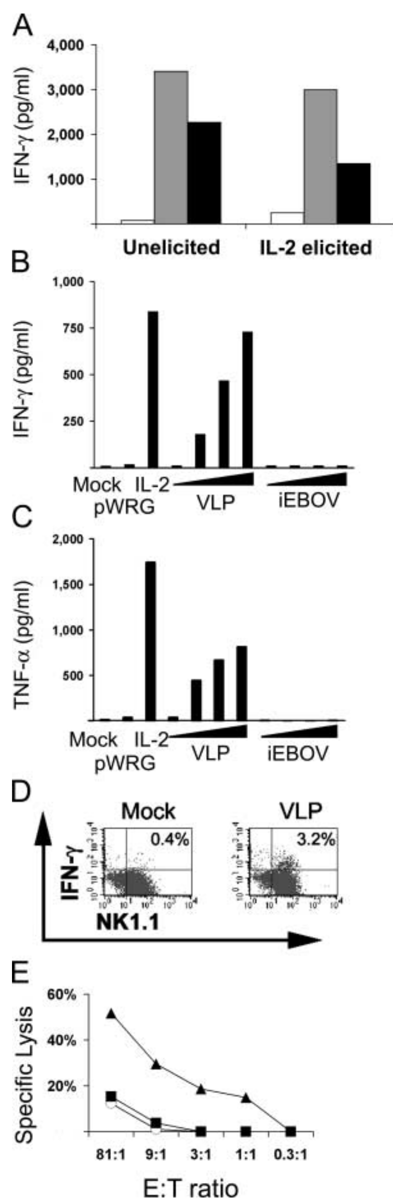

Figure 3. Ebola VLPs activate NK cells. (A) NK cells from the livers of unelicited or IL-2–elicited C57Bl/6 mice were incubated overnight with 10 μg of cell-free supernatants from pWRG vector-transfected cells purified on sucrose gradients (white bars, designated pWRG), 100 iU/ml of mouse IL-2 (gray bars), or 10 μg of VLP (black bars). The supernatants were assayed for IFN-γ by cytometric bead assay. (B and C) NK from the livers of IL-2–elicited C57Bl/6 mice were incubated overnight with media alone, IL-2, or increasing concentrations (0.5–50 μg) of VLPs or inactivated Ebola virus. The supernatants were assayed for (B) IFN-γ or (C) TNF-α. (D) NK cell preparations stimulated overnight with media or 10 μg of VLPs. The treated NK cells were stained for surface expression of NK1.1 and fixed, permeabilized, and stained for intracellular IFN-γ. The percent of viable lymphocytes (based on forward and side scatter), which were positive for both NK1.1 and IFN-γ, is indicated. The data in this figure represent three experiments of similar design and outcome. (E) NK cells were stimulated with VLPs for (■) 2 h or (▲) 18 h or (○) media alone. After the incubation period, the NK cells were added to ^{51}Cr-labeled YAC-1 cells at varying effector/target ratios as indicated. The amount of ^{51}Cr released into the supernatant was determined, and the percent specific release was calculated. Data are representative of at least two independent experiments.

firm that the NK cells were the main producers of IFN-γ. There was a considerable increase in the number of IFN-γ^+ and NK1.1$^+$ cells after VLP stimulation, as compared with NK cells incubated overnight in media alone (Fig. 3 D). These IFN-γ^+, NK1.1$^+$ cells did not express CD3 (unpublished data) and, thus, NK, not NK T, cells were specifically responsible for IFN-γ secretion. To show that this stimulation was the result of VLP preparations and not endotoxin contamination, VLPs or LPS were boiled or treated with polymyxin B, a compound that binds and neutralizes the biological activity of LPS (26), and the preparations were added to purified NK cells. Denaturation of VLPs by boiling, but not polymyxin B treatment, abrogated the NK cytokine responses; the opposite was true for LPS (unpublished data). NK cells stimulated with VLPs for 18 h, but not 2 h, specifically killed susceptible YAC-1 target cells (Fig. 3 E). These results show that Ebola VLPs induced strong NK cytotoxic activity, as well as cytokine and chemokine secretion.

NK Cell Responses to Ebola Virus. Ebola VLPs are morphologically and antigenically similar to live Ebola virus (Fig. 1 A and references 18, 23, 30). However, unlike VLPs, inactivated Ebola virus did not induce innate protection from Ebola virus infection or stimulate NK cell responses in vitro (Fig. 1 B). Therefore, we set out to determine if murine NK cells possessed the ability to respond to live Ebola virus. Unlike exposure to IL-2 or VLPs, live Ebola virus did not induce secretion of IFN-γ, MIP-1α, or TNF-α from NK cells (Fig. 4, A–C).

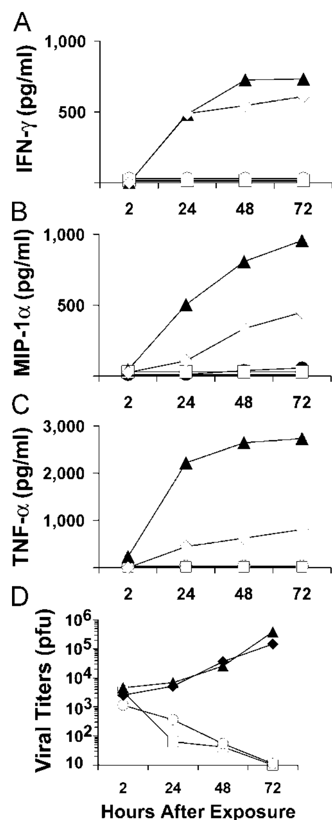

Figure 4. Ebola virus effects on murine NK cells. (A–C) The concentration of IFN-γ (A), MIP-1α (B), or TNF-α (C) in cell supernatants of NK cells exposed to 1 moi of Ebola virus-Zaire 95 (○) or –mouse adapted (●), 10 μg of VLPs (◇), 100 iU/ml of IL-2 (▲), or media alone (□) was determined over time using ELISA. (D) Viral titers in murine NK cells exposed to Ebola virus. Murine NK cells were infected with 1 moi of Ebola virus-Zaire 95 (○) or –mouse adapted (□). As a control, VeroE6 cells were infected with 1 moi of Ebola virus-Zaire (■) or –mouse adapted (◆). The cell-free supernatants were assayed for growth of Ebola virus using plaque assay at the indicated times. The data are presented as the number of PFU generated after exposure of 10^6 NK cells over time. These data are representative of three similar and separate experiments.

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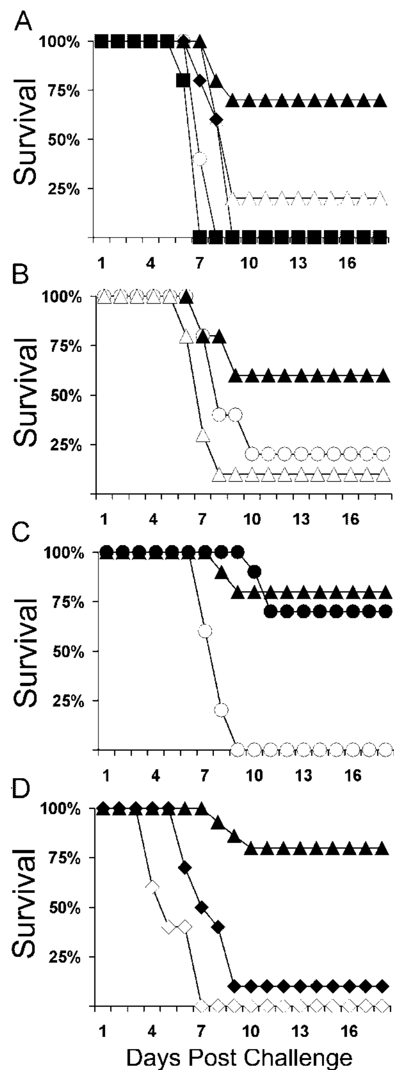

Figure 5. Perforin-dependent protection mediated by NK cells against Ebola virus. (A) NK cells from IL-2–treated C57Bl/6 mice were incubated overnight with 1 (△, $n = 5$) or 10 μg/ml (▲, $n = 20$) of VLPs, 50 μg/ml of inactivated Ebola virus (■, $n = 10$), 10 μg/ml of polyI:C (◆, $n = 5$), or media alone (○, $n = 10$). Naive recipient mice were injected with 5×10^6 treated NK cells and challenged 6 h later with 10 PFU of mouse-adapted Ebola virus. The results are presented on Meier-Kaplan survival curves. By a one-way Fisher's exact test, transfer of NK cells treated with 10 μg of VLPs, but not 1 μg of VLPs or 50 μg of inactivated Ebola virus, significantly increased the proportion of the mice surviving challenge ($P < 0.0001$) compared with mice receiving media-treated NK cells. (B) NK cells were isolated from the livers of IL-2–treated mice by negative selection. These highly enriched NK cell preparations were incubated overnight with (▲) VLPs or (○) media alone. Alternately, the NK cell preparation was depleted of NK1.1$^+$ cells using magnetic beads, and this NK cell–depleted (>90% reduction) population was stimulated with VLPs (△). After overnight incubation, the cell populations were injected into naive recipient mice ($n = 10$/group), and the mice were challenged 6 h later with 10 PFU of mouse-adapted Ebola virus. The results are presented as percent survival for each group and the survival curves were generated using data from two separate experiments with five mice per group. A significant increase in survival was observed in mice receiving the VLP-treated NK cells when compared with mice that received media-treated NK cells ($P < 0.0001$). In contrast, there was not a significant difference in survival between the mice receiving cell preparations depleted of NK cells and treated with VLPs, when compared with mice receiving media-treated NK cells ($P = 0.5891$). (C) NK cells were harvested from IFN-γ–deficient (C57Bl/6 background) mice. The NK cells were incubated overnight with VLPs (●) or media alone (○) and transferred to naive C57Bl/6 mice. As a control, NK cells from C57Bl/6 mice were incubated overnight with VLPs (▲) and transferred to naive recipient C57Bl/6 mice. The recipient mice were challenged with 10 PFU of Ebola virus and monitored for illness. The results are presented as percent survival for each group ($n = 10$), and the survival curves were generated using data from two separate experiments with five treated mice per group. A significant increase in survival was observed in mice receiving the VLP-treated NK cells isolated from IFN-γ–deficient or wild-type C57Bl/6 mice ($P = 0.0007$ or 0.0015, respectively) when compared with control mice that received media-treated NK cells. (D) NK cells were harvested from perforin-deficient (BALB/c background) mice and were incubated overnight with VLPs (◆) or media alone (◇). As a control, NK cells from BALB/c mice were incubated overnight with VLPs (▲). 5×10^6 stimulated NK cells were transferred to naive BALB/c mice by intraperitoneal injection. The recipient mice were challenged with 10 PFU of Ebola virus and monitored for illness. The results are presented as percent survival for each group ($n = 10$), and the survival curves were generated using data from two separate experiments with five treated mice per group. A significant increase in survival was observed in mice receiving the VLP-treated NK cells isolated from wild-type BALB/c mice ($P = 0.0007$) when compared with control mice that received media-treated NK cells. However, mice receiving VLP-treated NK cells from perforin-deficient mice did not have a significant increase in survival compared with control mice that received media-treated NK cells ($P = 0.5000$).

Several viruses, including human cytomegalovirus, HIV, and Epstein-Barr virus replicate efficiently in NK cells (31–34). To determine whether the lack of NK cell responses to Ebola virus were caused by Ebola virus infection of the NK cells, we determined the viral titers in supernatants of murine NK cells exposed to Ebola virus (multiplicity of infection [moi] = 1, Zaire 95 or mouse adapted). Ebola virus did not replicate in NK cells; in fact, the amount of live virus in the supernatants decreased during the 72 h after exposure to virus (Fig. 4 D). The inability of Ebola virus to replicate in NK cells was not due to death of the NK cells, as mock-infected and Ebola virus–infected NK cells had nearly the same viability after 3 d in culture (unpublished data). As expected, both viruses grew quickly to high titers in permissive VeroE6 cells (Fig. 4 D and references 19, 20). Neither wild-type Ebola virus–Zaire nor the mouse-adapted strain of Ebola virus stimulated cytokine secretion in NK cells nor replicated efficiently in murine NK cells, indicating the mouse-adapted Ebola virus does not differ drastically from the wild-type Ebola virus–Zaire in regards to the effects on NK cells (Fig. 4, A–C).

NK Cell–mediated Protection against Ebola Virus Is Perforin Dependent. Collectively, our observations prompted us to determine whether these functional responses of the VLP-exposed NK cells could reconstitute the short-term protection from Ebola virus observed in mice injected with VLPs. To do this, VLP-treated NK cells were transferred to naive mice, and the mice were challenged with Ebola virus. Animals treated with NK cells stimulated with a 10-μg dose of VLPs showed high survival rates (14/20, survivors/total) and even those mice that were treated with NK cells that had been stimulated with a low dose of VLPs developed enhanced protection against Ebola virus challenge (Fig. 5 A). In contrast, none of the mice receiving NK cells treated with either 50 μg/ml of inactivated Ebola virus, 10 μg/ml

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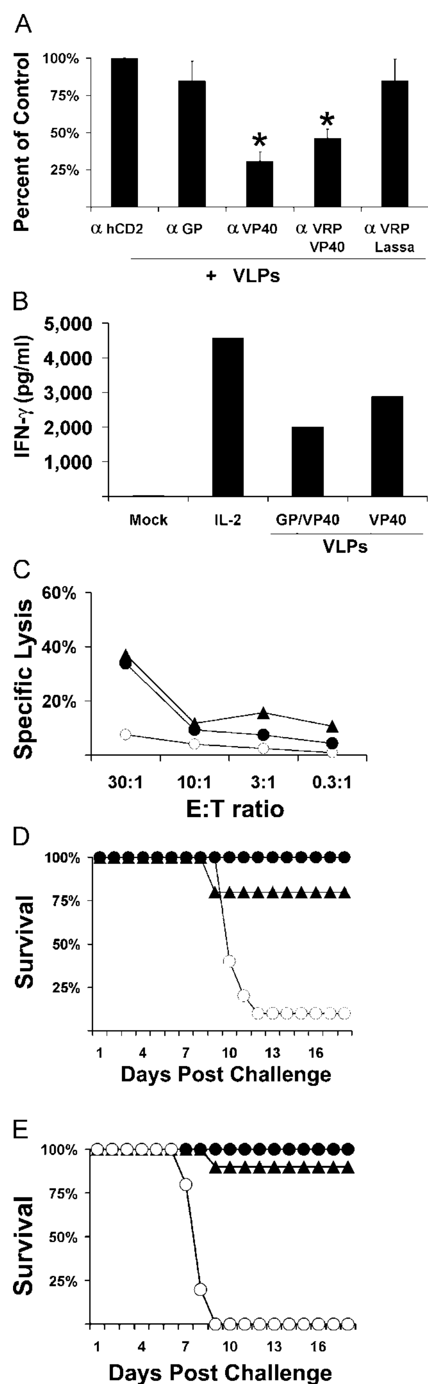

Figure 6. Ebola virus VP40 is sufficient to induce NK cell responses. (A) Antibodies, including either 30 μg of an irrelevant monoclonal to human CD2 (hCD2), a pool of three monoclonals against GP (αGP), or a monoclonal that recognizes VP40 (αVP40), 30 μl of sera from mice vaccinated with Venezuelan equine encephalitis replicon particles expressing VP40 (VRP-VP40), or Lassa virus N (VRP-Lassa), were preincubated for 1 h on ice with 10 μg of VLPs. Purified NK cells were incubated overnight with the antibody–VLP complexes, and the concentration of IFN-γ in the NK cell supernatants was determined. The data are shown as percentage of the control sample (range: 510–829 pg/ml), which was calculated by the following equation: [IFN-γ secretion with test antibody/ IFN-γ secretion with control antibody (hCD2)] × 100%. The graph shows the mean of three experiments with errors bars demonstrating the standard deviation from the mean of the three experiments. *, significant inhibition ($P < 0.05$) compared with the control culture as determined by paired Student's t test. (B) VLPs, made of GP and VP40, or VLP_{VP40} containing VP40 alone, were incubated with NK cells overnight, and the levels of IFN-γ in the NK cell supernatants were determined. Data are representative of four independent experiments. (C) NK cells were incubated with VLPs (▲), VLP_{VP40} (●), or media alone (○) overnight and added to ^{51}Cr-labeled YAC-1 cells at varying effector/target ratios. The amount of ^{51}Cr released into the supernatant was determined, and the percentage of specific release was determined. Similar results were obtained in two separate experiments. (D) NK cells were incubated overnight with 10 μg/ml of VLPs (▲), VLP_{VP40} (●), or media alone (○). Naive mice were injected intraperitoneally with 5×10^6 of the VLP- or media-treated NK cells and challenged 6 h later with 10 PFU of mouse-adapted Ebola virus. The results are presented as percent survival for each group ($n = 10$), and the survival curves were generated using data from two separate experiments with five treated mice per group. A significant increase in survival was observed in mice receiving the VLP- or VLP_{VP40}-treated NK cells ($P = 0.0027$ or 0.0001, respectively) when compared with control mice that received media-treated NK cells. (E) C57Bl/6 mice were primed with 10 μg of VLPs (▲), VLP_{VP40} (●), or PBS (○) 3 d before challenge with 10 PFU of mouse-adapted Ebola virus. The data are presented as percent survival for each group ($n = 10$), and the survival curves were generated using data from two separate experiments with five treated mice per group. A significant increase in the proportion of mice surviving was observed in mice treated with VLPs ($P < 0.0001$) or VLP_{VP40} ($P < 0.0001$) when compared with control mice injected with PBS.

polyI:C, or media alone survived (Fig. 5 A). Mice that failed to survive, but received VLP-stimulated NK cells, survived longer after Ebola virus infection than mice administered unstimulated NK cells (Fig. 5 A). To confirm that the NK cells, and not another cell type, were required for protection from Ebola virus infection, NK1.1$^+$ cells were depleted (>90% removed) from the standard NK cell preparation and the remaining cells in the preparation were transferred after overnight incubation with VLPs. The preparation containing NK cells, but not the NK1.1$^+$ cell–depleted preparation, protected animals from lethal Ebola virus infection (Fig. 5 B). VLP-stimulated NK cells from IFN-γ–deficient mice resulted in a high level of survival (Fig. 5 C), similar to NK cells from wild-type mice. In contrast, VLP-stimulated NK cells isolated from perforin-deficient mice did not elicit protection from Ebola virus infection (Fig. 5 D). Thus, although IFN-γ conventionally plays a major role in innate viral infection, this cytokine was apparently not involved in innate protection against Ebola virus; however, the protection was tightly connected to perforin-dependent cytotoxic activity of the NK cells treated with VLPs.

Ebola VP40 Is Sufficient to Induce NK Responses. The Ebola VLPs are enveloped particles, comprised of the GP and the matrix VP40, that bud from cellular lipid rafts (23). We sought to determine whether one of these viral components of the VLPs was responsible for the induction of NK responses. A single mAb against VP40, but not a pool of three mAbs against GP or irrelevant antibody (anti–human CD2), was able to block IFN-γ secretion by the VLP-stimulated NK cells (Fig. 6 A). Sera from mice vaccinated with VRP-encoding Ebola VP40 blocked IFN-γ secretion induced by the VLPs, whereas control sera from mice vaccinated with a VRP encoding the Lassa virus N had no effect (Fig. 6 A).

To further examine the role of VP40, we took advantage of the fact that expression of Ebola virus VP40 alone in

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mammalian cells also results in generation of VLPs (VLP_{VP40}), although with lower efficiency than with expression of both GP and VP40 (35, 36). NK cells stimulated overnight with VLP_{VP40} secreted cytokines, including IFN-γ (Fig. 6 B). Additionally, VLP_{VP40}-treated NK cells displayed cytotoxic activity against susceptible targets, similar to NK cells treated with VLPs (Fig. 6 C). When NK cells were stimulated overnight with VLP_{VP40} and transferred to naive mice, they fully protected mice from lethal challenge with Ebola virus infection (Fig. 6 D). Additionally, mice administered VLP_{VP40} 3 d before infection with mouse-adapted Ebola virus were completely protected from this lethal challenge (Fig. 6 E). These data suggest that the main viral protein involved in the innate immune responses to VLPs, including the NK-mediated protective effect, is the matrix protein VP40.

Discussion

We have established a model system to examine Ebola virus pathogenesis using hollow, genome-free VLPs. The VLPs swiftly induced effective protective immune responses in mice. This innate protection was dependent on NK cells because NK cell–deficient and NK cell–depleted mice were not protected from Ebola virus by the VLPs. NK cells exposed to VLPs secreted proinflammatory cytokines and chemokines and killed susceptible target cells. Furthermore, the transfer of VLP-activated NK cells was sufficient to elicit substantial protection against lethal filovirus infection in mice. The mechanism of innate protection against Ebola virus was not dependent on IFN-γ, but perforin was required. The protective effect of the VLP-induced NK cell activity was due mainly to the viral matrix protein VP40.

Functional changes in NK cells were not detected after exposure to live or inactivated Ebola virus. NK cells did not secrete cytokines, including IFN-γ, TNF-α, or MIP-1α, in response to Ebola virus. Similarly, our in vivo studies have suggested that Ebola virus infection of mice or monkeys does not activate significant NK cell responses (unpublished data). Ebola virus may actively interfere with or avoid innate immune responses, including NK responses (5, 6). Ebola virus GP has been proposed to modulate host adaptive immune responses (37). However, GP does not interfere with early innate immune responses, specifically NK cell responses, in the context of VLPs because protective immune responses are elicited by both VLPs and VLP_{VP40}. Ebola virus VP35 is the other known immune modulator and has been identified as an IFN antagonist. In Ebola virus–infected cells, VP35 blocks phosphorylation and dimerization of interferon regulatory factor 3, effectively preventing transcription of key antiviral genes (6, 38, 39). Although Ebola virus was not able to replicate efficiently in murine NK cells, it is possible that the virus was able to bind to, or enter, these cells and interfere with their response to the viral antigens through VP35 or other viral proteins. Although the mechanisms are unclear at this time, the virulence of Ebola virus may depend on its ability to evade or down-regulate the innate immune cell responses to viral infections, especially early responders such as NK cells. In fact, there is a specific loss of NK cells and a decrease in NK cell function after Ebola virus infection of primates (references 40, 41 and unpublished data). Together with our current findings, these data indicate a role for NK cells in the pathogenesis of Ebola virus.

Viral proteins are capable of directly inducing NK cell responses (29). Filovirus GPs represented the most likely candidates for interacting with NK cells directly, as the two other viral proteins known to directly induce NK cell responses are also GPs. The murine-activating receptor Ly49H directly recognizes a MCMV-encoded GP m157, which is an MHC-like molecule (29, 42). The NKp44 and NKp46 receptors on human NK cells interact with the influenza virus GP hemagglutinin via sialic acid side chains, leading to the NK cell–mediated lysis of influenza virus–infected cells (43, 44). In contrast, we found that the viral matrix protein VP40, and not Ebola virus GP, is critical and sufficient for the induction of innate, and specifically NK cell, responses to Ebola virus. Previously, Ebola virus GP has been presumed to be the only viral protein exposed on the surface of the virion. However, it is possible that VP40 is partially exposed on the virus surface. A recent paper indicated that mAb against the Marburg virus VP40 protein are capable of inducing complement-mediated lysis of infected cells (45). Crystallographic data show that VP40 can form octamers with a central pore, reminiscent of pore-forming toxins that insert into the plasma membrane (46). Furthermore, VP40 possesses integral membrane association characteristics and oligomerizes in the rafts of host cell membranes before driving virus particle formation (47, 48). Therefore, it is possible that VP40 is partially exposed on the surface of VLPs, and that this might be important for the stimulatory effect of these particles on innate immune cells. We propose that recognition of VP40 may be critical for alerting early, innate immune responses, whereas the immune responses to GP plays a more important role in the subsequent generation of protective adaptive immune responses.

In contrast to NK cells from wild-type C57Bl/6 mice, VLP-stimulated NK cells isolated from perforin-deficient mice failed to protect naive mice from lethal Ebola virus infection. Perforin-mediated NK cytotoxicity has a well-established role in tumor surveillance (49) and has a recognized, but less appreciated, role in viral infections (50, 51). Our data are in line with previous findings where control of HSV-1 infection in the eye and MCMV infection in the spleen of adult mice is mediated via a perforin-dependent mechanism (50–52). NK cell cytotoxic activity can be directly activated by receptor–ligand interactions or induced by exposure to cytokines including IFN-α/β, TNF-α, or IL-12 (11). However, it is not yet clear whether the cytotoxic activity of VLP-stimulated NK cells is a direct effect, or the result of secondary stimulation mediated by cytokine production. The production of cytokines such as IFN-α/β,

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IFN-γ, and TNF-α by NK cells is important for both the direct and indirect antiviral activity of NK cells (11). Treating NK cells with VLPs induced considerable secretion of TNF-α, IFN-γ, and other proinflammatory cytokines in vitro. The cytokine responses to viral antigens was not due to priming by IL-2 pretreatment of the mice, as NK cells from the livers of untreated C57Bl/6 mice secreted cytokines in a similar pattern to that secreted by IL-2–treated mice after exposure to VLPs (Fig. 3 A and not depicted). However, IFN-γ does not appear to be essential for the protective action of VLPs, as cells from IFN-γ knockout mice were fully capable of conveying protection.

NK cells are activated through a variety of ligand–receptor interactions (29). NK cells stimulated with VLPs did not induce changes in the levels of cell surface NK activating or inhibitory receptors, and we were also unable to identify a specific population of NK cells associated with the IFN-γ secretion (unpublished data). Furthermore, VLP-stimulated NK cells from BALB/c mice secreted cytokines in a similar manner to C57Bl/6 mice and protected against Ebola virus challenge when transferred to naive mice (Fig. 5 D and unpublished data). Therefore, VLP stimulation of NK cells is not restricted to a single mouse strain, and it is not related to the expression of Ly49H-activating receptor (53). It is possible that NK cell activation by VLPs may not be receptor-mediated, but may be mediated purely by cytokines or other unidentified mechanisms.

PolyI:C treatment of NK cells significantly increases protection against HSV-1 infection when compared with protection provided by untreated cells (15). In contrast, we found that polyI:C treatment of NK cells before transfer did not confer protection from Ebola virus infection (Fig. 5 A), indicating that nonspecific stimulation of NK cells is not sufficient for protection. In support of these findings, CpG treatment of the NK cell preparations before transfer did not protect naive mice from Ebola virus challenge (unpublished data), further suggesting that activation of APCs in NK cell preparations could not account for the observed protection. Therefore, the protection provided by VLP-treated NK cells appears to be mediated by VLP-specific responses, although we do not understand the mechanisms of action at this time.

We were concerned that NK T cells contributed to the biological responses of our VLP-exposed cellular preparations. However, the NK cell–enriched preparations did not contain $CD3^+$ NK T cells, but were contaminated with eosinophils (<10%), $B220^+MHC$ class II^+ cells (<3%) that could be macrophages, DCs or B cells, and a small number of $CD5^+$ T cells (<2%). Depletion of $NK1.1^+$ cells from the cell preparations before transfer abrogated innate protection from Ebola virus, suggesting that contaminating APCs, eosinophils, or other lymphocytes were not required for innate responses to Ebola virus. Nonetheless, it may be that VLPs are taken up by DCs or macrophages, which in turn activate the NK cells or that VLPs are rapidly processed and presented by the APCs to B or T cells. In contrast, exposure to inactivated Ebola virus does not activate or mature murine DCs (18) and, thus, likely does not efficiently prime secondary lymphocyte responses. We have shown previously that both B and T lymphocytes are activated transiently 2–3 d after challenge in the lymph nodes of VLP-vaccinated mice (18). Changes in early T cell activation markers, including CD25, CD43, and CD69, are not detectable until at least 48 h after injection in lymph nodes, spleen, or peritoneal cavity and, thus, do not exactly correlate with the rapid protection observed in our current work within 1 d after injection (reference 18 and unpublished data). In this paper, we have shown a critical involvement of NK cells in innate protection against Ebola virus infection; however, at this time, we cannot rule out the contribution of other cell types, including DCs, B, and T cells.

The innate immune system provides early surveillance and control of viral infections. In this paper, we show that the innate immune response, specifically NK cells, can mediate rapid and complete protection against lethal Ebola virus infection. These observations represent a key advance in understanding the requirements for protective immunity against Ebola virus infection. The identification of NK cells as critical mediators of early protection against Ebola virus infection are an important step forward in the identification of prophylactic and therapeutic interventions against filovirus and other incapacitating acute viral infections. Although the exact application of these findings to therapeutics in treating Ebola virus–infected primates and humans is unclear at this time, therapeutic agents that bolster the innate immune response, including activation of NK cells, should be the target of future studies.

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