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Cutting Edge: Impairment of Dendritic Cells and Adaptive Immunity by Ebola and Lassa Viruses

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Acute infection of humans with Ebola and Lassa viruses, two principal etiologic agents of hemorrhagic fevers, often results in a paradoxical pattern of immune responses: early infection, characterized by an outpouring of inflammatory mediators such as TNF-α, IL-1β, and IL-6, vs late stage infections, which are associated with poor immune responses. The mechanisms underlying these diverse outcomes are poorly understood. In particular, the role played by cells of the innate immune system, such as dendritic cells (DC), is not known. In this study, we show that Ebola and Lassa viruses infect human monocyte-derived DC and impair their function. Monocyte-derived DC exposed to either virus fail to secrete proinflammatory cytokines, do not up-regulate costimulatory molecules, and are poor stimulators of T cells. These data represent the first evidence for a mechanism by which Ebola and Lassa viruses target DC to impair adaptive immunity. The Journal of Immunology, 2003, 170: 2797–2801.

Ebola virus, a member of the Filoviridae family, and Lassa virus, a member of the Arenaviridae family, represent two important etiological agents of viral hemorrhagic fevers (1, 2). Despite their disparate taxonomies, the diseases caused by both viruses share striking similarities, and are characterized by a rapidly progressive febrile illness with capillary leakage, often resulting in extensive mucosal and s.c. hemorrhage (3, 4). In its severe form, the illness frequently progresses to a septic shock-like syndrome with multisystem failure culminating in death. Careful studies of pathogenesis in infected humans have not been performed because of the remote location of human outbreaks and the accompanying lack of facilities for rigorous experimentation. Consequently, very little information is available about the cellular and molecular mechanisms that mediate the pathogenesis of these infections.

Data from in vitro experiments and animal models suggest that both Ebola and Lassa viruses infect macrophages and endothelial cells early in infection (5–7). Infection of these cell types, two principal players of the innate immune system, are believed to act as critical triggers for the rapid and uncontrolled secretion of inflammatory mediators (2, 3, 8, 9). However, despite the systemic release of inflammatory mediators that occurs following infection with Ebola or Lassa, severe or fatal diseases are often associated with a generalized suppression of adaptive immunity, as evidenced by the low specific Ab (3) and poor cellular immunity (8, 10). The cellular and molecular mechanisms that underlie these seemingly paradoxical immune states are unknown.

Dendritic cells (DC) occupy center stage as the most efficient APC in the immune system and are scattered throughout the body, including the portals of virus entry where they reside in an immature form (11–14). Immature DC are capable of decoding and integrating signals from invading microbes and ferry this information to naive T cells in the secondary lymphoid organs, undergoing a maturation process en route. There, the mature DC present this information to T cells, thus launching an immune response. DC also play pivotal roles in modulating the strength and quality of the immune response (11–14). Given these pivotal roles, there is an urgent need to understand how Ebola and Lassa viruses can manipulate DC functions to influence the outcome of these diseases. This information is of vital importance in understanding the pathogenesis of Ebola or Lassa and in devising novel therapeutic strategies. Our results show that these viruses readily infect and replicate in DC. In striking contrast to their effects on macrophages and endothelial cells (5, 6, 15), our data suggest that infection with either Ebola or Lassa viruses fails to activate DC, thereby interfering with their ability to initiate an adaptive immune response. Taken together, these data represent the first evidence for a mechanism by which Ebola and Lassa viruses target DC and Ag presentation to disarm the adaptive immune system and may offer an explanation for the immune suppression that is associated with these hemorrhagic fevers.
Materials and Methods

Viruses
A viral isolate (Mayinga strain, Vero E6, pass 2) from the 1976 outbreak of Ebola Zaire was used in these experiments. Ebola virus was purified by centrifugation of Ebola-infected Vero E6 on a 20–60% sucrose gradient at 30,000 rpm for 16 h in a Ti-41 rotor (Beckman-Coulter, Fullerton, CA) (16). The band containing the concentrated virus was aspirated and washed twice by pelleting in PBS (30,000 rpm), titered by plaque assay, and stored in liquid nitrogen (17, 18). A Lassa virus (Niakhar strain) stock was prepared by amplification of a human isolate from Sierra Leone on Vero E6 cells. After 3 days of incubation, the virus was harvested (10,000 rpm, 30 min) and the cell slurry was pelleted (10,000 rpm; 20 min) and aliquots of the supernatant containing the virus were stored in liquid nitrogen (18). Virus titers were determined by plaque assay. DC were infected at a multiplicity of infection (moi) of 1 (Ebola) or 0.5 (Lassa). Gamma-irradiated Ebola and Lassa aliquots were used as mock controls (5 × 10^5 rad of gamma radiation).

Cell culture and in vitro infection with viruses
PBMC were obtained by apheresis from healthy human donors. Monocyte-dec- derived DC (MDDC) were generated by standard methods as described previ- ously (19). Briefly, CD14+ monocytes purified from blood PBMCs using mAbs against CD14 (Dynal Biotech, Norway) were cultured in six-well plates (1 × 10^6/well) in RPMI 1640 (Life Technologies, Gaithersburg, MD) with 10% FBS (low endotoxin FCS; HyClone, Logan, UT) for 6 days in the presence of GM-CSF (100 ng/ml) and IL-4 (5 ng/ml; both from PeproTech, Rocky Hill, NJ) in a 5% CO2 incubator as previously described (19). This resulted in enrichment for CD11c+CD14+HLA-DR+ MDDC (>90%). On day 6 of culture, immature MDDC were infected in vitro with either Ebola or Lassa virus (live or inactivated) by mixing virus stocks directly with pelleted aliquots of 0.5 × 10^6 cells at the desired moi and incubation with rock- ing at 37°C for 45 min, followed by one wash in medium. Cells were then cultured at 0.5 × 10^6 cells/ml in 48- or 96-well tissue culture plates (Corning, Corning, NY). Control cells were treated with medium alone, or an equivalent dose of gamma-irradiated Ebola or Lassa virus or PBS (1–2 μg/ml Escherichia coli LPS; Sigma-Aldrich, St. Louis, MO). Supernatants and cells were harvested after 48 h of incubation at 37°C in 5% CO2. All infections were done in a biosafety level 4 laboratory at the Centers for Disease Control and Prevention, and samples were gamma-irradiated before processing for other as- says in a biosafety level 2 laboratory.

Phenotyping of DC cells
Activation of MDDC was evaluated by surface staining for CD86, CD80, or CD40. Briefly, after 48 h of incubation under various conditions, cells were washed twice with cold PBS (pH 7.2) containing 0.5% BSA (Sigma-Aldrich) (wash buffer) and incubated with fluorescent labeled Abs to CD11c, CD14, HLA-DR, and CD86 (BD PharMingen, San Diego, CA) for 30 min at 4°C. Cells were then washed and pelleted twice with wash buffer (1000 rpm) and fixed with 10% for- malin for 4 days. Cells were then analyzed for surface expression of these markers by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). Cells that were CD11c+“HLA-DR−” were analyzed for expression of CD80, CD86, or CD40.

Immunofluorescent Ab staining for viral proteins
Infection of cells with Ebola or Lassa virus was confirmed by indirect immunoflu- orescence assays using reagents reported previously (18, 20). Viral proteins were detected by incubation with a pool of seven anti-Ebola mAbs (1:100 in PBS with 5% milk and 0.5% Tween 20) or rabbit polyclonal anti-Lassa serum (1:100 in the same buffer), followed by anti-mouse or anti-rabbit FITC conjugates (Bio-Rad, Hercules, CA) and examined under UV light for the presence of viral proteins.

Focus-forming unit assays
Virus was quantified in the supernatant of cells by a focus-forming unit assay as described (21). Abs against the cytokines were coupled to a single set of carboxy- lated microspheres using a two-step carbodiimide coupling procedure. Micro- sphere sets were mixed so that they would all be at approximately the same concentration. Two hundred microspheres were analyzed for each test. Samples (diluted 1:2 and 1:100) were added to duplicate wells of black flat-bottom half-well plates (Costar, Acton, MA). The plates were covered with adhesive foil and incubated for 1 h with constant agitation. Biotinylated Abs were diluted in stor- age buffer (PBS pH 7.2, 1 mg/ml BSA-0.05% sodium azide) to a final concen- tration of 1 μg/ml (mAbs: IL-10, IL-8, TNF-α, IFN-γ, IL-4, IL-6, and monocyte chemotaectant protein-1 (MCP-1)) or 5 μg/ml (polyconal Abs: IL-1β, macrophage-inflammatory protein (MIP)-1α, MIP-1β, and RANTES). Ten microliters of the Ab mix were added to each well, the foil cover was replaced, and the plate was incubated for 4 h with constant agitation. Streptavidin-conjugated PE was diluted in PBS-Tween to a final concentration of 5 μg/ml, and 100 μl was added to each well. Samples were analyzed on a Luminex 100 analyzer (Luminex, Austin, TX). Serial 3-fold dilutions of human recombinant cytokines were run on each plate to produce standard curves. Data was analyzed using PRISM analysis software (GraphPad, San Diego, CA).

Mixed lymphocyte reaction
Allogenic naive CD4+CD45RA+ T cells were purified using the MACS CD4+ cell isolation kit (Miltenyi Biotec, Auburn, CA). MDDC incubated in vari- ous conditions for 48 h were mixed in 96-well plates with various DC:T cell ratios. Cells were cultured at 37°C with 5% CO2 for 4 days and pulsed with [3H]thymidine (1 μCi/well; NEN, Boston, MA) for 16 h. Plates were gamma-irradiated to inactivate mitotic activity, and cells therein were harvested onto glass fiber filters. Scinti- tion (moi) of 1 (Ebola) or 0.5 (Lassa). Gamma-irradiated Ebola and Lassa ali- quots were used as mock controls (5 × 10^5 rad of gamma radiation).

Results

Ebola and Lassa viruses infect human MDDC
After 6 days of culture with GM-CSF and IL-4, immature MDDC constituted >90% of the cell population (data not shown). As reported previously (19), these cells were character- istically CD1a+, HLA-DR+, CD14−, CD80−, and CD86− (data not shown). To determine whether Ebola and Lassa vi- ruses could productively infect human MDDC, they were in- fected in vitro with either Ebola or Lassa virus (live or inacti- vated). The survival of DC as various times after infection was determined by trypsin blue staining (Fig. 1A). Neither Ebola nor Lassa viruses caused significant cell death in the first 4 days of culture (Fig. 1A). After 4 days, ~20–30% of cells were dead as determined by trypan blue staining. Immunofluorescence as- says for viral proteins (Ebola gp, nuclear protein, and viral protein 40, and all Lassa viral proteins) demonstrated that both Ebola and Lassa had infected significant numbers of MDDC by 48 h (Fig. 1B). Furthermore, quantitation of the viruses in su- pernatants of MDDC at 48 h by plaque assays confirmed that both Ebola and Lassa virus established productive infections with increasing viral titers: an increase of ~3 logs in Ebola titers (from log titer 4.0 at the start of culture to 7.1) and ~1.5 log in Lassa viral titers over the amount of virus used in infection (from log titer 4.0 at the start of culture to 5.5) (Fig. 1C). These results indicate that Ebola and Lassa viruses can infect and re- plicate within MDDC and are therefore capable of directly interfer- ing with cellular function.

Ebola and Lassa viruses do not induce proinflammatory cytokines or costimulatory molecules on human MDDC
Given that Ebola and Lassa viruses can infect MDDC, we were interested in determining whether this resulted in alteration of MDDC function. To address this question, we measured the secretion of inflammatory cytokines and chemokines by MDDC following infection with Ebola and Lassa viruses. In addition to TNF-α, IL-1β, IL-6, and IFN-α, cytokines that have been proposed to play a crucial role in the inflammation follow- ing infection with Ebola (21, 22) and Lassa (6, 9) viruses, we
also measured the secretion of IL-8, MIP-1α, MIP-1β, and MCP-1, chemokines that are likely to be involved in recruitment of macrophages, neutrophils, and T cells. The interaction of DC with diverse microbial stimuli, such as LPSs, CpG bacterial DNA, or viral heat shock proteins is known to induce the production of several proinflammatory cytokines (11–14). Unexpectedly, we found that MDDC infected or stimulated by either live or killed Ebola and Lassa viruses did not secrete TNF-α, IL-1β, IL-6, IL-10 (Fig. 2A), or IL-2, IFN-γ, or IL-12 (data not shown), as would be expected after a typical inflammatory stimulus, such as LPS (14). This effect on MDDC is in striking contrast to the effect of these viruses on macrophages (5, 6), and to the effects of other viruses such as dengue (23, 24) on DC, although several viruses such as the measles virus has been shown to inhibit DC function in a similar manner (25, 26). Interestingly, the secretion of certain chemokines (notably IL-8 and MCP-1) was significantly higher with Ebola or Lassa virus, compared with LPS (Fig. 2A), suggesting a selective negative effect of these viruses on the induction of the proinflammatory cytokines.

One of the defining properties of mature MDDC is the expression of costimulatory molecules, an attribute that endows these cells with the ability to efficiently stimulate naive T cells (11–14, 19). We wondered whether infection with Ebola or Lassa viruses could modulate the expression of costimulatory molecules on DC. To address this question, we compared the expression of CD86, CD80, and CD40 on Ebola- or Lassa-infected MDDC with LPS-stimulated MDDC (a stimulus known to up-regulate the expression of these costimulatory molecules). Forty-eight hours after infection, Ebola virus and, to a lesser extent, Lassa virus failed to induce the expression of CD86, and other costimulatory molecules on DC (Fig. 2B). In contrast, DC stimulated with LPS showed a significant up-regulation of costimulatory molecules. Taken together, these results suggest that infection of MDDC with Ebola and Lassa viruses, or culture of MDDC with inactivated Ebola or Lassa viruses, results in a selective impairment of proinflammatory cytokines and costimulatory molecules (Fig. 2B). However, the viruses do appear to induce chemokines such as IL-8 and MIP-1α.

**Ebola and Lassa viruses impair MDDC-mediated adaptive immunity**

The initiation of an adaptive immune response to an invading pathogen is a critical function of DC (11–14). To determine whether, in these experiments, failure of Ebola and Lassa viruses to induce proinflammatory cytokines and costimulatory molecules on DC had direct consequences on the induction of adaptive immunity, we examined the effect of infection with Ebola and Lassa on their ability to induce the proliferation of naive CD4+CD45RA+ T cells in an MLR. As seen in Fig. 3, DC...
infected with live Ebola or Lassa viruses fail to induce the proliferation of allogenic T cells. In fact, the level of proliferation induced by such DC is even lower than that observed with unstimulated DC. In contrast, DC matured with LPS elicit significant levels of proliferation, as observed previously (11–14).

To confirm that the inhibition of T cell proliferation was not simply the consequence of lysis of MDDC infected with Ebola and Lassa viruses, we assessed the survival of DC on days 2, 4, and 6 after infection, because T cells were pulsed on day 6 of culture. As shown in Fig. 1A, there was a modest decrease in

**FIGURE 2.** Secretion of inflammatory and anti-inflammatory mediators and activation of MDDC in response to Ebola and Lassa viruses. *A.* Cytokine and chemokine secretion by MDDC (0.5 × 10⁶ cells) cultured for 48 h with live or inactivated Ebola or Lassa viruses, or LPS (1 μg/ml). *B.* Expression of the costimulatory molecules CD86, CD80, and CD40 on MDDC following Ebola and Lassa virus infection. MFI, Mean fluorescence index.

**FIGURE 3.** Impaired function of DC infected with Ebola or Lassa virus. DC were infected with Ebola or Lassa, washed, and then cultured with naive, allogeneic CD4⁺ T cells for 5 days. Uptake of [³H]thymidine is shown in the graph as cpm on the y-axis, and MDDC pretreatment condition is shown on the x-axis. Bars and error bars represent the means of quadruplicate cultures from each of four donors and the SEM. TC, T cells alone; Med, medium.
surviving cells on day 6 (80% survival with Ebola, 75% survival with Lassa infection, and ~60% with LPS treatment), but between days 2 and 4 postinfection, >80% of the original cell numbers were alive irrespective of infection. Thus, at the time of initiation of T cell proliferation by DC (days 2–4 of culture), there were no significant differences in the number of DC in the infected and uninfected wells, implying that DC mortality is unlikely to be responsible for the observed inhibition of T cell proliferation by Ebola and Lassa virus.

Discussion

Systemic release of inflammatory mediators has been considered to be the hallmark of viral hemorrhagic fevers (7–9, 21, 27). Much of the data supporting a critical role for inflammatory cells has come from studies of macrophages, one of the early targets of viral replication for both Ebola and Lassa. Interactions between Lassa virus and DC have not been reported to date, and although Ebola virus has been shown to be capable of infecting A549 and H9251 cells, a substantial difference in the number of DC in the infected and uninfected wells (data not shown). The mechanism of this difference is clearly an area that deserves attention. Finally, understanding the mechanisms by which either Ebola or Lassa viruses fail to activate DC and adaptive immunity may identify novel strategies of augmenting immune responses.

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