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Protection from lethal infection is determined by innate immune responses in a mouse model of Ebola virus infection

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Abstract

A mouse-adapted strain of Ebola Zaire virus produces a fatal infection when BALB/cj mice are infected intraperitoneally (ip) but subcutaneous (sc) infection with the same virus fails to produce illness and confers long-term protection from lethal ip rechallenge. To identify immune correlates of protection in this model, we compared viral replication and cytokine/chemokine responses to Ebola virus in mice infected ip (10 PFU/mouse), or sc (100 PFU/mouse) and sc "immune" mice rechallenged ip (10^6 PFU/mouse) at several time points postinfection (pi). Ebola viral antigens were detected in the serum, liver, spleen, and kidneys of ip-infected mice by day 2 pi, increasing up to day 6. Sc-infected mice and immune mice rechallenged ip had no detectable viral antigens until day 6 pi, when low levels of viral antigens were detected in the livers of sc-infected mice only. TNF-α and MCP-1 were detected earlier and at significantly higher levels in the serum and tissues of ip-infected mice than in sc-infected or immune mice challenged ip. In contrast, high levels of IFN-α and IFN-γ were found in tissues within 2 days after challenge in sc-infected and immune mice but not in ip-infected mice. Mice became resistant to ip challenge within 48 h of sc infection, coinciding with the rise in tissue IFN-α levels. In this model of Ebola virus infection, the nonlethal sc route of infection is associated with an attenuated inflammatory response and early production of antiviral cytokines, particularly IFN-α, as compared with lethal ip infection.

Keywords: Ebola virus; Filovirus; Cytokines; Viral kinetics; Innate immunity; Protective immunity; Pathogenesis; Interferons

Introduction

Ebola viruses are members of the family *Filoviridae* and are the cause of a severe hemorrhagic fever in humans in Africa. The virus reappears in infrequent epidemics that are characterized by human-to-human transmission resulting in a febrile illness often associated with hemorrhagic manifestations in the skin and mucosal membranes. The first recognized epidemics of Ebola hemorrhagic fever (EHF) were simultaneous outbreaks in Zaire and Sudan in 1976, in which there were a total of 610 cases with a case fatality rate of 90% (in the Congo) and ~55% (in Sudan) (Anonymous, 1978a,b). Major outbreaks of EHF with large case numbers and similar mortality rates have occurred subsequently in 1979 (Sudan), 1995 (Zaire), and 2000 (Uganda) (Anonymous, 2001; Baron et al., 1983; Khan et al., 1999). In each of these outbreaks, the public health priorities associated with this highly transmissible disease have understandably mandated the institution of measures aimed primarily at

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containing the epidemic, with little opportunity for research into the biology of the virus or to identify factors that correlate with survival or protection against severe disease. The small numbers of survivors and their location in remote Central-East Africa have been a further obstacle to well-designed studies of EHF.

In human infections, replication of virus in the primary target cells (macrophages and endothelial cells) occurs early in the disease, often during the asymptomatic phase (Feldmann et al., 1999; Schnittler and Feldmann, 1998; Zaki and Goldsmith, 1999). Further organ dissemination of the virus follows, accompanied by symptomatic illness with fever, skin rashes, and mucosal hemorrhagic lesions (in more severe disease). There is a prominent gastrointestinal component to the illness, with abdominal pain, nausea, vomiting, and diarrhea, often with melena. This phase is recognized in the laboratory by the appearance of viral antigen in the serum, titers of which rise rapidly as the illness progresses. In surviving patients, the appearance of anti-Ebola antibodies typically occurs in the second week of illness followed by the disappearance of viral antigens from circulation (Ksiazek et al., 1999a). The clearance of the virus is likely to be mediated by antigen-specific cellular responses (Baize et al., 1999; Gupta et al., 2001b; Sullivan et al., 2000; Wilson and Hart, 2001). In fatal cases, however, the illness proceeds rapidly with a progressive rise in circulating virus titers accompanied by shock and multiorgan failure and hemorrhage from skin and mucosal sites occurs in a substantial proportion of fatal cases.

Studies done during and after outbreaks have succeeded, to some extent, in defining the virology and antibody response kinetics of human Ebola virus infections (Ksiazek et al., 1999a), but the absence of a sophisticated technical/laboratory infrastructure in EHF- endemic areas has made it impossible to study many aspects of immunity and pathogenesis of the disease in humans. However, a recently developed mouse model of Ebola virus infection, using a mouse-adapted strain derived from the prototype (human) Ebola Zaire 1976 isolate, may greatly facilitate the study of several aspects of the disease (Bray et al., 1998).

The mouse-adapted virus produces disease in all strains of mice tested, including BALB/c, C57/BL6, and outbred strains (Bray et al., 1998). While there are many similarities between the disease produced by the mouse-adapted virus in mice and the disease in nonhuman primates and humans (Gibb et al., 2001), a unique feature of this mouse-adapted virus is the difference in outcome when adult mice are infected via different routes. Mice infected by intraperitoneal (ip) injection uniformly develop symptomatic infection with rapid progression to death and 100% mortality (Bray et al., 1998), even with a very low inoculum [<1 plaque-forming unit (PFU)]. However, the same virus, at doses as high as 10^6 PFU per mouse, when injected subcutaneously (sc), causes no illness or death in all the strains of mice tested (Bray, 2001). The remarkable dichotomy in the course of infection following virus entry by different routes suggested that host factors, particularly differences in the innate immune response, were likely to be involved (Bray, 2001).

The goal of this study was to examine kinetics of Ebola virus growth and to identify potential mechanisms of pathogenesis that determine different outcomes in mice infected by two routes (sc and ip). With these data, we aim to better understand the pathogenesis of acute Ebola virus infections and to identify the correlates of immune protection in this murine model that could guide the development of effective Ebola vaccines. We show that the sc route of infection is characterized by a slow rise in the virus load, apparently allowing clearance, presumably by antiviral humoral and cellular responses, whereas the rapid dissemination and multiplication of virus after ip inoculation appears to circumvent or escape the same clearance mechanisms. Inflammatory responses, characterized by tumor necrosis factor (TNF)-α and monocyte chemotactic protein (MCP)-1 production, appear early after ip infections, correlating with increasing virus loads, whereas sc infection is associated with early production of interferon (IFN)-α and IFN-γ, potent antiviral cytokines. The IFN-α response in sc-infected mice was associated with protection from rechallenge as early as 48 h postinfection (pi). These data identify some correlates of protection against Ebola virus in this model and suggest a balance between protective immune responses and replication of virus that might be manipulated to influence the outcome.

Results

Attenuated viral growth associated with sc infection when compared with ip infections

Intraperitoneal infection of adult BALB/cj mice with 10 PFU ($\sim$300 times the LD_{50}) of the mouse-adapted Ebola virus resulted in a uniformly fatal infection: 100% mortality within 7 days (data not shown), whereas sc inoculation with 100 PFU resulted in no mortality. Furthermore, sc-infected mice did not lose weight nor exhibit any overt signs of illness (weight loss, ruffled fur, huddling, or inactivity), as seen in mice infected ip. When mice surviving sc infection were challenged by the ip route (10^6 PFU/mouse) 3 weeks after the initial challenge, they again exhibited no signs of illness, and 100% survived for the 21-day observation period. As expected, the naïve control group that received the same dose of Ebola virus ip all died by day 7. Thus, sc-infected mice acquire solid resistance against rechallenge by a route (and dose) that normally results in fatal infection.

We examined viral antigen levels at different time points during acute infection to determine if viral replication occurred in sc-infected mice and to elucidate the relationship between route of infection and viral growth. As seen in Fig. 1, viral antigen (AG) titers in liver, spleen, kidney, and serum following a primary infection differed between ip-

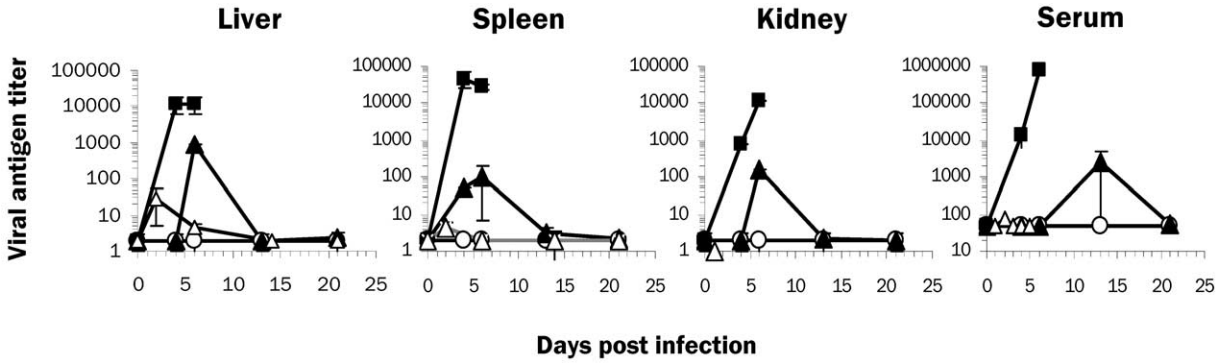

Fig. 1. Virus antigen kinetics in BALB/cj mice infected with a mouse-adapted Ebola virus ip, sc, or immune mice challenged ip. Viral antigen titers in the liver, spleen, kidney, and serum of mice infected with Ebola virus ip ($n = 3$ per time point; ■), sc ($n = 3$ per time point; ▲), sc-infected mice rechallenged ip ($n = 3$ per time point; △), or uninfected mice ($n = 2$ per time point; ○). Symbols represent the mean viral antigen titer, measured by ELISA (see Materials and methods) and error bars denote standard errors. Sera were serially fourfold diluted starting at a dilution of 1:50, and tissue homogenates (10% w/v in PBS) were also fourfold diluted serially, starting at a dilution of 1:2. *y*-axes show AG titers, and *x*-axes show the day postinfection. The sensitivity threshold of the assay ranged from 1:2 (for livers, spleens, and kidneys) to 1:40 (serum). All mice in an observation group infected ip died by day 7 postinfection. Differences in antigen titers between ip-infected and sc-infected as well as immune rechallenged mice were statistically significant on days 5 and 6 ($P < 0.05$; ANOVA). Data are representative of two similar experiments.

and sc-infected mice. After ip infection, virus AG titers rose rapidly by day 4 and continued to rise until day 6, with death occurring on days 6–7 postinfection, whereas sc infection resulted in a delayed appearance of viral AGs in all tissues, with peak titers that were 100- to 1000-fold lower than those observed in ip-infected mice and returning to baseline by day 15 (for organs) and day 21 for serum. A notable observation in the sc-infected mice was that virus AG first appeared in the spleen (day 4 postinfection), then in the liver and kidney on day 6, and much later in serum, where a peak was reached on day 14 (Fig. 1).

As noted, mice infected sc demonstrated solid resistance to ip rechallenge. Such resistance could be due to neutralization of the virus at the point of entry (preventing any viral growth), or to clearance of the virus after a period of replication. To determine if virus replication did occur in the immune mice, we measured virus AG titers in the serum and tissues of mice challenged ip 3 weeks after sc infection. These experiments revealed that very low titers of virus AG were detectable on day 2 post-ip challenge in the serum, liver, and spleen, but not in the kidney. Virus AG titers were 4–5 logs lower than in ip-infected mice and 1–2 logs lower than in sc-infected mice (Fig. 1). In each tissue, viral AG disappeared by day 6, indicating complete clearance of the virus. Thus, in "immune" mice virus replication was not completely prevented but rather was strongly attenuated and the virus was rapidly cleared after rechallenge with a dose that is normally lethal via the ip route.

To identify some correlates of protective immunity in immune mice, we compared both innate and adaptive immune responses in mice infected with Ebola virus via the sc and ip routes. With regard to adaptive immunity, antibodies have been proposed to play a protective role in several animal models of Ebola virus infection (Gupta et al., 2001b; Parren et al., 2002; Takada et al., 2003; Wilson et al., 2000; Xu et al., 1998). Lethal (ip) infection with the mouse-adapted Ebola virus was associated with a lack of detectable anti-Ebola IgG up to the time death (data not shown). In contrast, sc-infected mice had high titers of anti-Ebola IgG on day 13 postinfection that remained essentially unchanged up to the end of their 21-day observation period. Thus, in experiments in which immune mice were rechallenged ip, they had high anti-Ebola IgG titers at the time of rechallenge on day 21 (Gupta et al., 2001b). Further, IgG titers were boosted approximately fourfold by day 8 postchallenge by the ip route in immune mice (data not shown). These data are in agreement with previous studies that have shown that anti-Ebola antibodies are able to protect against fatal infection in some animal models (Gupta et al., 2001b; Parren et al., 2002; Wilson et al., 2000). These results are also consistent with clinical observations: human survivors have high anti-Ebola IgG titers, whereas fatal cases have low or undetectable titers of antibodies at the time of death (Baize et al., 1999; Ksiazek et al., 1999a).

Nonlethal infection is associated with an early induction of innate immune responses

Inhibition of innate antiviral mechanisms appears to play an important role in viral replication and pathogenesis in Ebola virus infections (Basler et al., 2000; Bray, 2001; Bray et al., 2002; Harcourt et al., 1998, 1999). To ascertain the role of innate immune responses in resistance to Ebola virus infections in mice made immune by sc infection, we measured IFN-α levels in tissues and sera of mice infected sc or ip, and sc immune mice challenged ip (Fig. 2). We measured the levels of the mediator in target tissues (liver, spleen, and serum) during two phases of infection: (i) the early phase, immediately following exposure to the virus, before detectable virus replication (the first 48 h of infec-

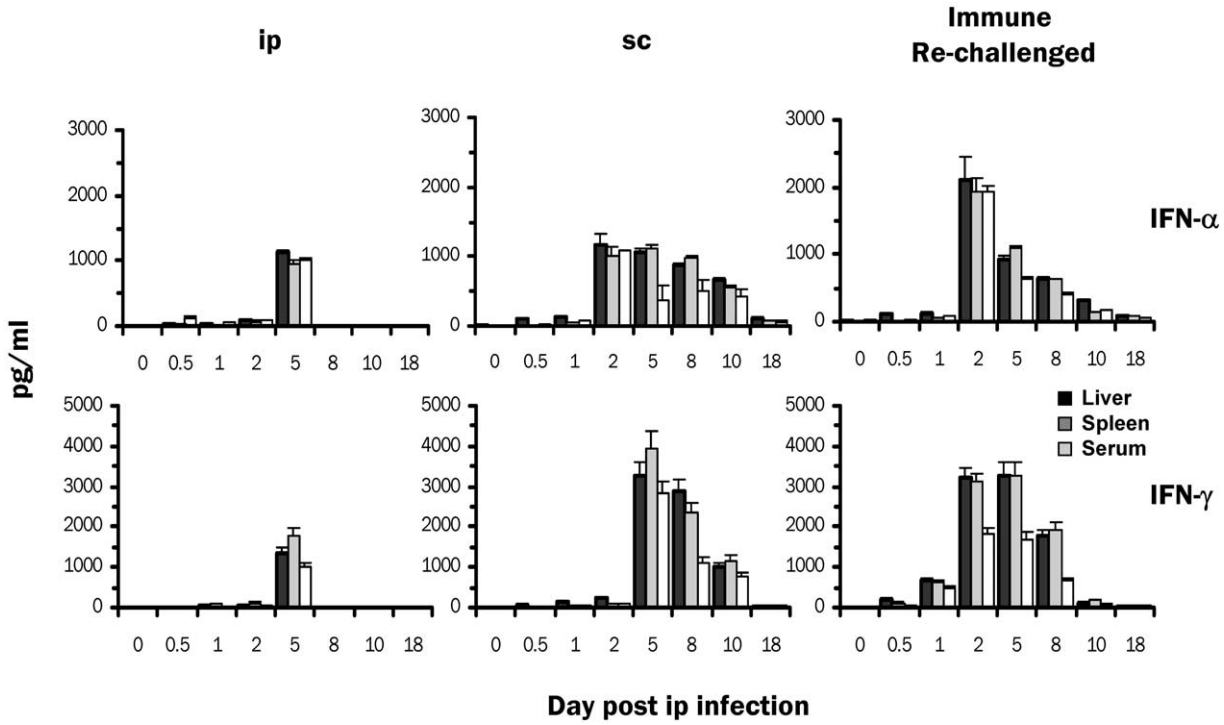

Fig. 2. Nonlethal infection with Ebola virus is associated with early innate immune responses. BALB/cj mice were given a lethal ip infection (10 PFU/mouse), a nonlethal sc infection (100 PFU/mouse) with Ebola virus, or an ip challenge (10^6 PFU/mouse) 3 weeks after an sc infection, as labeled. The levels of IFN-α (top graphs) and IFN-γ (bottom graphs) were determined in homogenates (10% w/v in PBS) of livers (■), spleens (▒), and serum (□) from mice sacrificed at the indicated time points. Homogenates of livers from uninfected mice had <20 pg/ml of each cytokine. Data from separate experiments for the early time points (0–2 days postinfection) and late time points (5–18 days postinfection) are combined. Each bar denotes the mean and standard error for three mice. Each experiment was performed twice with similar results.

tion); and (ii) the late phase, when virus replication is easily detected (days 2–18). In the studies of the early phase of infection, inactivated virus produced a slight and transient elevation of IFN-α in tissues and sera in all the groups (ip, sc, and immune rechallenged ip) returning to baseline within 48 h (peak levels <200 pg/ml in all tissues). Mice infected ip with live virus had low levels of IFN-α (<100 pg/ml) until day 5 when modest levels of the cytokine were detected (959–1121 pg/ml average in the liver, spleen, and serum; Fig 1). At this late stage of infection, virus antigen levels were extremely high (titers $> 10^5$). In striking contrast, tissue and serum IFN-α levels in sc- infected mice were significantly elevated as early as 2 days postinfection, and even higher levels were found in the sc immune mice upon rechallenge. Subcutaneously infected mice had a sustained elevation of IFN-α in all tissues from day 2 until day 10 (500–1250 pg/ml) and the levels then declined to baseline by day 18 (Fig. 2). Immune mice had a similar rapid rise in IFN-α after ip challenge, but a more rapid decline, reaching baseline values by day 10, a pattern that differed considerably from that seen in mice infected ip (Fig. 2). Thus, clearance of the virus correlated with an early innate antiviral response, sustained throughout the period of viral replication, as evidenced by detection of viral antigens (Fig. 1).

The levels of the antiviral T cell derived cytokine IFN-γ in tissues after Ebola virus infection were determined as a surrogate for adaptive (cellular) immune responses. Inactivated virus induced low levels of IFN-γ in ip and sc "mock"-infected mice (<100 pg/ml in homogenate in all tissues). IFN-γ was detectable after 1 day in the spleen of immune mice rechallenged with inactivated virus (272 $\pm$ 117 pg/ml of homogenate; Fig 2); however, this transient increase declined by day 2 postinoculation and was significantly less than levels seen in mice infected with live virus. Intraperitoneal infection with live virus resulted in undetectable levels of IFN-γ until day 5 postinfection, when modest levels of the cytokine were detected in all tissues (Fig. 2). In contrast, sc infection was associated with high levels of IFN-γ on day 5 that were sustained up to day 10 postinfection, returning to baseline by day 18. In striking contrast, immune mice rechallenged ip with Ebola virus had a rapid IFN-γ response, within 48 h of infection, that was sustained until day 8 postinfection, returning to baseline by about day 10, earlier than in sc-infected mice (Fig. 3). Thus, significant adaptive cellular immune responses, represented by IFN-γ, appeared only at late stages of infection in ip-infected mice and during the phase of viral clearance in sc-infected mice and in rechallenged mice.

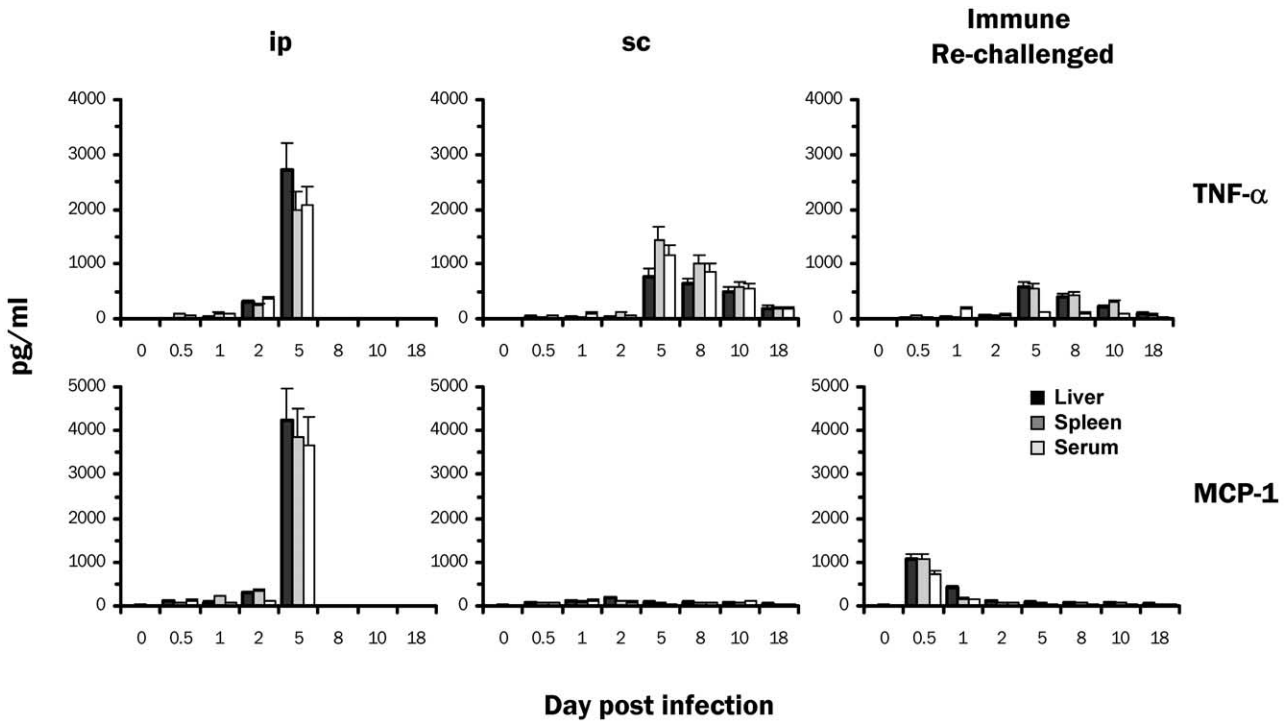

Fig. 3. Lethal Ebola virus infections are associated with secretion of inflammatory cytokines and chemokines. BALB/cj mice were infected in groups of 3 with sc (100 PFU of Ebola virus per mouse), ip (10 PFU per mouse), or immune mice were challenged ip (10^6 PFU per mouse) as labeled and the levels of TNF-α (top graphs) and MCP-1 (bottom graphs) were determined in homogenates of livers (■), spleens (▒), and serum (□). Homogenates of livers from uninfected mice had <20 pg/ml of TNF-α and <40 pg/ml of MCP-1 (not shown). Separate experiments were done for the early time points (0–2 days postinfection) and late time points (5–18 days postinfection), and the results of both are plotted together. Each bar denotes the mean and the error bar, the standard error for a group. Data are representative of two experiments for each phase of infection.

Induction of inflammatory responses is associated with lethal ip infection

Since monocytes and tissue macrophages are known to be important targets of Ebola virus (Gibb et al., 2001; Sanchez et al., 2001; Schnittler and Feldmann, 1999; Zaki and Goldsmith, 1999), we examined the levels of representative monocyte-derived or macrophage-activating cytokines (TNF-α) and chemokine (MCP-1) involved in the recruitment of macrophages and T cells into sites of infection. These mediators are induced by filovirus infection of both human and murine macrophages (Gupta et al., 2001a; Schnittler and Feldmann, 1999; Stroher et al., 2001) and are suspected to play an important role in the pathogenesis of Ebola virus in human and nonhuman primates (Hensley et al., 2002; Hutchinson et al., 2001; Schnittler and Feldmann, 1998; Villinger et al., 1999). As with IFN-α and IFN-γ, we determined the patterns of production of these mediators in target tissues during the early (days 0–2) and late (days 5–18) phases of infection.

We found very little sustained induction of these cytokines by inactivated virus (data not shown). In the early phase, ip injection of inactivated virus was associated with a rise in TNF-α levels in the liver (30 ± 4 pg/ml), spleen (118 ± 10 pg/ml), and serum (79 ± 9 pg/ml), whereas sc inoculation resulted in a transient but minimal rise in TNF-α (<100 pg/ml) in all tissues that returned to baseline by 48 h postinfection (data not shown). MCP-1 responses to inactivated virus were higher than the corresponding TNF-α responses, reaching peak levels of 205 and 266 pg/ml in the homogenates of livers and spleen, respectively, at 12 h postinfection only in immune ip rechallenged mice, but returning to baseline levels by 2 days postinfection. After live virus infection by both sc and ip routes, TNF-α levels were elevated in all tissues on day 5, with levels in ip-infected mice (1980–2730 pg/ml) being significantly higher than in sc-infected mice (780–1430 pg/ml; $P < 0.05$; Fig. 3). Interestingly, TNF-α levels were significantly lower in immune-rechallenged mice, with a peak also at around day 5. TNF-α levels correlated with the levels of virus AGs in ip- and sc-infected mice, but in immune mice after rechallenge, the levels were elevated well after day 2, the peak of virus replication (Fig. 1). We used two approaches to determine if TNF-α plays a direct role in the pathogenesis of fatal (ip) Ebola infections. First, survival of mice in which TNF-α and/or lymphotoxin (LT)-β activity was blocked by treatment with fusion proteins comprising the LT-β receptor (R) or TNF-αR combined with an Fcγ molecule following ip infection with Ebola virus was compared to that of control mice and found to be identical (data not shown). Second, mice genetically deficient in expression of TNF-αR (p75) were challenged ip with Ebola virus. These mice were

also found to have an identical survival pattern as that of congenic controls (data not shown). From these preliminary data, it appears that blockade of the high TNF-α levels in mice with fatal infections does not alter the course of infection, suggesting that it is not the primary mediator of a poor outcome in ip infections.

MCP-1 levels in tissues were elevated in a pattern similar to TNF-α in ip-infected mice with high levels on day 5 postinfection when viral AG levels were also markedly elevated. In contrast, sc-infected mice had very low levels of MCP-1 throughout the course of infection, and immune mice rechallenged ip had an early, modest, and transient elevation of MCP-1 that returned to baseline within 2 days (Fig. 3). Taken together, these data confirm the notion that secretion of these inflammatory cytokines and chemokines accompanies the uncontrolled viral replication in macrophages that occurs in ip-infected mice. In sc-infected and immune mice rechallenged ip, where viral replication is modest or minimal, inflammation is correspondingly mild or absent.

Innate resistance to rechallenge is dependent on virus dose and interval after sc infection

Given that significant levels of IFN-α were found in the serum as early as 48 h after sc infection, we attempted to determine whether this strong IFN response was involved in protection of mice against an ip virus challenge. First, we determined the interval after sc infection that was necessary for the development of resistance. Mice infected sc with 10^6 PFU Ebola virus and then challenged ip with 10 PFU of Ebola virus after 18 or 24 h had a mortality of 90 and 80%, respectively (Fig. 4A). However, all the mice challenged at 48 h survived. Second, we determined the dose of sc-inoculated virus required for development of resistance to rechallenge at 48 h (Fig. 4B). The resistance to rechallenge was found to be dependent on the dose of virus used in the initial sc infection. All mice infected sc with 10^6 PFU survived an ip rechallenge 48 h later, but mice given 10^5 PFU of virus sc had a 50% mortality and those that received $\leq 10^4$ PFU virus sc had $\geq$90% mortality following ip rechallenge 48 h later (Fig 4B). It should be noted that the 48 h interval after the sc infection, which appears to be necessary for protection against ip rechallenge, coincides with the induction of IFN-α after sc infection (Fig 3) and is too short an interval for the appearance of AG-specific effector CD8 T cells. Thus, the early resistance, which appears to be dependent on innate immunity, correlated with the production of IFN-α.

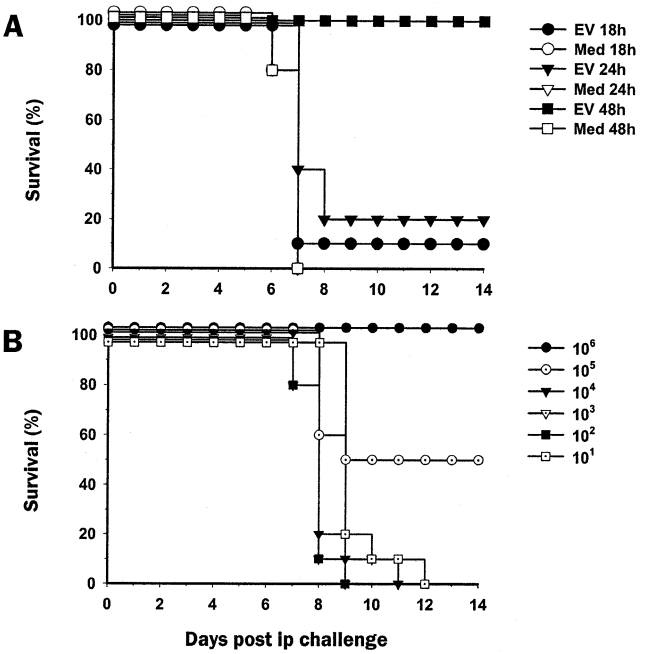

Fig. 4. Dependence of early resistance to Ebola virus in sc-infected mice on innate immunity. (A) Groups of five BALB/cj mice were infected sc with 10^6 PFU Ebola virus per mouse and rechallenged ip (10 PFU of Ebola virus per mouse) after varying intervals from 18–48 h as indicated by the key. Rechallenged mice were observed for 14 days and survival or death was recorded. (B) Groups of five BALB/cj mice were infected sc with different doses of Ebola virus as indicated by the key and rechallenged ip with 10 PFU of Ebola virus per mouse after 48 h. Mice were observed for 14 days. The *y*-axis represents percentage of mice surviving. EV: Ebola virus; Med: medium alone.

Discussion

The mouse model of Ebola virus infection has unique features that make it possible to study susceptibility and resistance to lethal infection in the same animal model and to perform studies of the immunobiology of the virus and testing of antiviral agents in ways that are not possible to do in humans and nonhuman primates. Human infections are rare, occurring in sporadic epidemics during which research studies and testing of antiviral agents are difficult. Nonhuman primates, although excellent models for studies of pathogenesis and therapy for Ebola virus, are expensive and require specialized laboratories. A tremendous advantage of the mouse model is the availability of a wide variety of reagents and genetically altered mice that can be used to study many aspects of the immune responses to the virus. Indeed, studies using knockout mice for investigation of pathogenesis and protective immunity against Ebola virus are already under way in several laboratories (M. Bray and M. Gupta, personal communication).

Previous studies with this mouse model of Ebola virus infection have shown that the route of inoculation determines the outcome of infection (Bray, 2001; Gibb et al., 2001). However, these studies did not report on the kinetics of viral growth or on the type of immune response generated by sc and ip infections. In this study, we have demonstrated that viral loads are lower in sc- than in ip-infected mice and that the virus is completely cleared within 14 days of infection. The complete recovery of sc-infected mice suggests that the apparently slower replication and dissemination of

the virus may allow sufficient time or opportunity for the host to generate or expand clearance mechanisms. Other studies have implicated both humoral and cellular immunity as effector mechanisms in the clearance of Ebola virus in the mouse (Gupta et al., 2001b; Rao et al., 1999, 2002; Wilson et al., 2001; Wilson and Hart, 2001). Our data showing that in sc-infected mice, viral AG appears around day 6 postinfection in the serum (Fig. 1) and, later on, in the liver and spleen support the notion that viral replication does occur but is both slower and lower in magnitude in sc than in ip infections. Our antigen data in ip-challenged immune mice (Fig. 1) indicate that, although the protection in immune mice is solid, virus replication does occur initially, but rapid clearance is achieved without detectable clinical symptoms.

The difference in the kinetics of TNF-α and MCP-1 production in sc- and ip-infected mice is an important finding in this study. These (and other) inflammatory mediators have been proposed to play a role in the pathogenesis of Ebola virus in humans (Leroy et al., 2000; Sanchez et al., 2001; Villinger et al., 1999) and in the mouse model (Bray, 2001; Gibb et al., 2001; Gupta et al., 2001a; Peters and LeDuc, 1999), and are likely involved in recruitment of immune effector cells (macrophages, CD8 cells, NK cells) to the sites of viral replication. TNF-α may also participate in the initiation of lymphocyte apoptosis, a prominent feature of Ebola virus infections in humans (Baize et al., 1999, 2000) that has also been demonstrated in nonhuman primate models (Geisbert et al., 2000) and possibly in the massive lymphocytolysis seen in mice (Gibb et al., 2001). In the case of ip-infected mice, however, the pattern of early TNF-α secretion in serum and tissues, with a later, and modest antiviral response (IFN-α and -γ) after virus had replicated to high titers (Figs. 1 and 3), suggests that a strong inflammatory response may portend a fatal outcome.

The role of IFN-α and IFN-γ in protection as revealed by our data appears to be complex. Although the levels of these cytokines were elevated in the two groups that cleared the virus (sc-infected and immune mice), the design of these experiments did not allow us to distinguish between a causative role for these cytokines and an alternative role as surrogates for some other viral clearance mechanism. However, data from other studies do ascribe a direct role to IFN-α in protection against Ebola virus in this murine model (Bray, 2001; Bray et al., 2002). The first study by Bray demonstrated that a nonlethal sc infection with the mouse-adapted Ebola virus could be converted into a lethal infection in IFN-α receptor knockout mice and upon blockade of IFN-α with antibodies (Bray, 2001). The author concludes that induction of IFN-α by the mouse-adapted virus following sc infection may be a critical factor that allowed the virus to be cleared in the murine host.

We and others have previously demonstrated that Ebola virus inhibits the release of IFN-α induced by double-stranded RNA (Basler et al., 2000; Gupta et al., 2001a; Harcourt et al., 1998, 1999). Here, we have shown that the IFN-α response to the virus differs depending on the route of infection. Infection by the ip route results in a weak IFN-α response to Ebola virus early in infection. These observations are consistent with earlier data in isolated macrophages showing that Ebola virus inhibits IFN-α responses to double-stranded RNA (Gupta et al., 2001a). In contrast, sc infection results in an early and rapid production of IFN-α that is associated with protection from fatal disease. These interesting observations, taken together with the data showing that the initial rise in IFN-α levels in tissues occurs after 24 h (Fig. 2), as well as the knockout experiments described above (Bray, 2001), suggest that type I IFN-mediated viral clearance plays a role in this early resistance. The reason for this difference in IFN-α responses between the two routes of infection is not clear, but may be related to the cells of the innate immune system that first interact with the virus. The mechanisms involved in clearance of Ebola virus have not been characterized to date but accumulating evidence from this mouse model indicates that CD8 T cells play a critical role (Wilson and Hart, 2001). Clearance mediated by CD8 T cells likely occurs at a later stage of infection, only in animals that survive. Our data and those reported from other models of viral infections suggest that IFN-α may not only be important in the early control of viral growth, but also has an important regulatory role in generation of effector CD8 T cells responses later in infection, as has been proposed for other viruses (Biron, 1998, 1999; Cousens et al., 1999).

An important difference between the sc and ip route of infection that may explain differences in outcome is the antigen-presenting cell populations involved in initiating the immune response. Subcutaneous infection involves peripheral dendritic cell (DC) populations, such as Langerhans cells or lymphoid interdigitating DC as the initial presenting cells, whereas ip infection probably results in presentation to T cells via peritoneal macrophages and macrophage-derived DC. Differences in viral kinetics between the two routes of infection may, therefore, be due to differences in the antigen-presenting cell populations or to the cytokine milieu associated with peripheral DC and peritoneal macrophages (Banchereau et al., 2000; Pulendran et al., 2001). It is tempting to speculate that differences in the ability of Ebola virus or its proteins to inhibit early secretion of type I interferons in macrophages and DC may play a role in the differential clearance of the virus in sc and ip infections.

In this study, we explored an unusual feature of the mouse model of Ebola virus infections, the differences in outcome between two routes of infection, to identify potential mechanisms of pathogenesis and protective immunity against Ebola virus. The observed delay and attenuation of viral growth in sc-infected mice suggest a dynamic balance between the virus and host clearance mechanisms leaning in favor of the host in the sc route of infection. The mechanisms of viral growth attenuation in sc infection are unclear, but accumulating evidence suggests that they may operate through the action of cellular antiviral defenses, such as type

I IFNs. Exploitation of the mouse model to investigate these antiviral defenses will undoubtedly advance our understanding of the importance of innate and adaptive immune mechanisms in the development of protective immunity against this virus.

Materials and methods

Virus, cells, and media

We used a mouse-adapted Ebola virus in our experiments because pilot experiments demonstrated that a human isolate of Ebola Zaire 1976 strain failed to produce infections in mice with doses as high as 10^6 PFU per mouse (data not shown). The mouse-adapted strain of Ebola virus was derived from Ebola Zaire 1976 by serial passage through progressively older suckling mice, followed by plaque purification as described (Bray et al., 1998). For all our experiments, we used a stock of the mouse-adapted strain after one Vero E6 cell passage (titer 5×10^7 PFU/ml). Vero E6 cells (monkey kidney cell line, ATCC Vero C1008, CRL1586) were propagated in modified Eagle's medium (EMEM), 2% fetal bovine serum, glutamine (2 mM, Life Technologies, Gaithersburg, MD), streptomycin (100 μg/ml, Life Technologies), and penicillin (100 U/ml, Life Technologies). In some experiments, aliquots of the viral stocks were gamma-irradiated (5×10^6 rad in a Gammacell 220, Nordion, Canada) and used as inactivated controls. Infectious material and animals were handled in a Biosafety Level (BSL) 4 laboratory at the Centers for Disease Control and Prevention (CDC), Atlanta, GA. All the samples brought out from the BSL 4 laboratory were gamma-irradiated (5×10^6 rad) before further processing in BSL 2 and 3 facilities. This inactivation process has been previously demonstrated not to affect the proteins measured in the assays used (Mahanty et al., 1999).

Mice

Female BALB/cj mice (aged 6–8 weeks) were obtained from commercial suppliers (Jackson Laboratory, Bar Harbor, ME and Harlan Sprague–Dawley, IN). All mice were allowed to acclimate to the BSL4 laboratory conditions for 3–4 days before use in our experiments. Data shown are representative of experiments repeated at least twice, using groups of three mice. Animal use was in strict adherence to the institutional guidelines for animal care at the CDC and the U.S. Army Medical Research Institute for Infectious Diseases.

Infection procedure

Lethal Ebola virus infections were produced by ip inoculation of 10 PFU of mouse-adapted Ebola virus (in 0.2 ml of PBS), and nonlethal infections by 100 PFU of mouse-adapted Ebola virus given sc. Subcutaneously infected immune mice (referred to hereafter as immune mice) were generated by infection with 100 PFU of live Ebola virus in 0.2 ml of PBS on the back below the neck (divided into two inocula to avoid leakage of the inoculum from dense sc tissue) as previously described (Bray et al., 1998). In experiments focusing on adaptive immunity, mice were challenged by ip inoculation of 10^6 PFU of live Ebola virus 21 days after sc infection. In experiments focusing on innate immunity, we rechallenged the mice ip with 10^6 PFU of Ebola virus at various intervals ranging from 12 to 48 h post sc infection with different doses (range 1–10^6 PFU/mouse). After infection, ear-tagged mice were caged in groups of 3, checked for survival daily, and weighed on alternate days for at least 21 days.

Processing of tissues

For antigen and cytokine assays, 10% (w/v in PBS) homogenates of gamma-irradiated tissues were prepared. Cellular debris was pelleted by microcentrifugation and the supernatant was used in assays as described below.

Quantitation of virus by antigen detection (ELISA) assay

Titers of Ebola viral antigens in sera and in homogenates of organs were determined by a capture ELISA as described (Ksiazek et al., 1992, 1999b). Briefly, 96-well plates (Fisher Scientific, Atlanta, GA) were coated overnight at 4°C with either a mixture of seven monoclonal mouse antibodies (MAbs) raised against VP40, GP, and NP from Ebola Zaire 1976 and Ebola Sudan 1976 or normal mouse ascitic fluid (for control wells). Tissue homogenates and serum samples were serially diluted (fourfold) in 5% PBS-skim milk with 0.1% Tween 20 (blocking buffer), added to the wells in duplicate, and incubated at 37°C for 1 h. Captured AG was detected by polyclonal anti-Ebola Zaire serum produced in rabbits and detected with a goat anti-rabbit HRP conjugate (Kirkegaard and Perry, MD) and ABTS substrate (Kirkegaard and Perry). The optical density (OD_{410}) for each sample in control wells was subtracted from the OD of the corresponding dilution of the same sample in wells coated with anti-Ebola MAbs to derive a corrected OD. The highest dilution of sample that resulted in a corrected OD of $\geq$0.1 corresponded to the viral AG titer of the sample. Viral AG titers were found to have a log-linear relationship with viral PFU counts (data not shown) for values $\geq$ 300 PFU. We used viral AG titers to compare levels of virus in sera and tissues.

Chemokine and cytokine assays

Sera and homogenates of livers and spleens were assayed, after inactivation with gamma-irradiation (5×10^6 rad), using commercially available ELISA kits for the cytokines TNF-α (R&D Systems, Minneapolis, MN), IFN-α

(PBL, Inc., Princeton, NJ), IFN-γ (R&D), and MCP-1 (R&D) according to the manufacturers' instructions. For the purpose of comparison, values were expressed in pg/ml by interpolation of sample OD values on standard curves generated with recombinant cytokines/chemokine.

Statistics

Comparisons of virus AG titers and cytokine levels in sera and tissue between experimental groups were done using the Student's *t*-test on log-transformed data. Animal weights and survival data were compared by ANOVA. Differences between groups for categorical data were analyzed using the Fisher's exact test. P -values of <0.05 were used as the cutoff for statistical significance.

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