Seoul virus suppresses NF-κB-mediated inflammatory responses of antigen presenting cells from Norway rats

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Abstract

Hantavirus infection reduces antiviral defenses, increases regulatory responses, and causes persistent infection in rodent hosts. To address whether hantaviruses alter the maturation and functional activity of antigen-presenting cells (APCs), rat bone marrow-derived dendritic cells (BMDCs) and macrophages (BMDMs) were generated and infected with Seoul virus (SEOV) or stimulated with TLR ligands. SEOV infected both DCs and macrophages, but copies of viral RNA, viral antigen, and infectious virus titers were higher in macrophages. The expression of MHCII and CD80, production of IL-6, IL-10, and TNF-α, and expression of Ifnβ were attenuated in SEOV-infected APCs. Stimulation of APCs with poly I:C prior to SEOV infection increased the expression of activation markers and production of inflammatory cytokines and suppressed SEOV replication. Infection of APCs with SEOV suppressed LPS-induced activation and innate immune responses. Hantaviruses reduce the innate immune response potential of APCs derived from a natural host, which may influence persistence of these zoonotic viruses in the environment.

Keywords:
Dendritic cells
Hantavirus
Innate immunity
Interferon
Macrophage
MHC
Rodent
Toll-like receptor
TNF
Viral persistence

Introduction

To date, more than 50 hantaviruses (Family: Bunyaviridae) have been found worldwide (Klein and Calisher, 2007), with each hantavirus presumably having coevolved with or adapted to specific rodent or insectivore hosts (Plyusnin and Morzunov, 2001; Arai et al., 2008; Ramsden et al., 2009). As a result of the close relationship between hantaviruses and hosts, hantavirus infection of rodent reservoirs results in a persistent infection that is devoid of overt pathological disease (Meyer and Schmaljohn, 2000a; Easterbrook and Klein, 2009). Currently, the mechanisms mediating persistence of hantaviruses in their natural hosts remain unknown. We hypothesize that virus-induced suppression of antiviral and proinflammatory responses may be one mechanism contributing to hantavirus persistence in reservoir hosts. Data from male Norway rats infected with their species-specific hantavirus, Seoul virus (SEOV), reveal that in tissues (e.g., the lungs) that support elevated virus replication, the expression and production of antiviral (e.g., IFN-β, IFN-γ, and Mx2) and proinflammatory (e.g., IL-1β, IL-6, and TNF-α) proteins are significantly higher during the persistent phase than during the acute phase of SEOV infection (Easterbrook et al., 2007; Easterbrook and Klein, 2008b, 2008c; Hannah et al., 2008).

Upregulation of regulatory T cell activity contributes to hantavirus persistence in rodents (Easterbrook et al., 2007; Schountz et al., 2007). In the lungs of male Norway rats, the frequency of CD4+ CD25+ FoxP3+ regulatory T cells, expression of FoxP3 mRNA, and production of TGF-β are significantly higher during the persistent phase than during the acute phase of SEOV infection (Easterbrook et al., 2007; Easterbrook and Klein, 2008b, 2008c). Furthermore, inactivation of regulatory T cells reduces the amount of SEOV RNA in the lungs and the duration of virus shedding in saliva (Easterbrook et al., 2007). In deer mice infected with Sin Nombre virus (SNV), the expression of Tgfβ in CD4+ T cells isolated during the persistent phase of infection is significantly higher than is the expression in CD4+ T cells isolated during the acute phase of infection (Schountz et al., 2007). The mechanisms mediating induction of regulatory T cells in reservoirs for hantaviruses remain elusive. In addition to host-mediated mechanisms, virus-mediated alterations affect persistence of hantaviruses. Terminal nucleotide deletions of the L segment of SEOV are correlated with persistence and...
reduced virus replication in Vero E6 cells (Meyer and Schmaljohn, 2000b). Thus, SEOV persistence in rats likely reflects both host- and virus-associated mechanisms.

In contrast to reservoir species, spillover of hantaviruses to humans causes severe morbidity and mortality. Depending on the virus species, human infection results in two observable clinical diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) (Klein and Calisher, 2007). Although the disease outcomes vary, both HFRS and HCPS are associated with enhanced vasculature permeability in target organs due to viral infection of vascular endothelial cells, monocytes, and macrophages (Nagai et al., 1985; Pensiero et al., 1992; Temonen et al., 1993). One prevailing hypothesis for the pathogenesis of HFRS and HPS is that disease is caused by immune-mediated effector mechanisms targeting infected cells (Pensiero et al., 1992; Sundstrom et al., 2001; Easterbrook and Klein, 2008a; Schonrich et al., 2008). Accordingly, studies have shown high frequencies of virus-specific CD8⁺ T cells and excessive levels of pro-inflammatory cytokines, such as TNF-α, IFN-β, and IL-6, in patients infected with hantavirus (Linderholm et al., 1996; Markotic et al., 1999; Mori et al., 1999; Kilpatrick et al., 2004).

Activation of T cells relies on the proper functioning of antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages. Many viruses can infect DCs and modulate their effector mechanisms often leading to either viral clearance or viral persistence in the infected host (Larsson et al., 2004). Hantaan virus (HTNV) infects human DCs with no apparent cytopathic effects (CPE) or impairment of effector functions (Rafferty et al., 2002). The surface expression of ICAM-1, CD80, CD86, MHC I, and MHC II, the production of TNF-α and IFN-β, and the ability to stimulate proliferation of naïve T cells in vitro are increased in HTNV-infected DCs as compared with mock-infected DCs. Hantavirus infection may serve as a maturation signal to activate immature DCs in humans, eliciting an excessively strong adaptive immune response that underlies disease pathogenesis (Schonrich et al., 2008). Hantavirus also infect and replicate in macrophages (Nagai et al., 1985; Temonen et al., 1993; Khaiboullina et al., 2000; Cebalo and Markotic, 2007; Markotic et al., 2007; Easterbrook and Klein, 2008b) resulting in increased production of antiviral proteins (e.g., IFN-α and MXA) and chemokines (CXCL8) in cells from humans (Temonen et al., 1995; Cebalo and Markotic, 2007). Monocytes and macrophages isolated from humans and infected with hantaviruses in vitro differentiate into a DC-like phenotype, increasing the antigen presenting capabilities of these cells which leads to viral clearance but also could contribute to the immune-mediated tissue damage seen in infected humans (Markotic et al., 2007).

Although the effects of hantavirus infection on DCs and macrophages isolated from humans have been studied, the immunological outcome of hantavirus infection of APCs from rodents has not been reported for any reservoir species and may provide insights into the innate immune mechanisms mediating the ability of hantaviruses to persist and not cause disease in rodent hosts. In the present study, we utilized bone marrow-derived DCs (BMDCs) and bone marrow-derived macrophages (BMDMs) to evaluate the effect of SEOV infection on the function of APCs derived from Norway rats. We hypothesized that hantavirus infection would impair the functional activity of DCs and macrophages from Norway rats, possibly down-regulating the innate antiviral and proinflammatory responses necessary for viral clearance and increasing the regulatory phenotype (i.e., production of IL-10 and TGF-β) of these cells.

**Results**

**TLR ligands induce maturational changes in rat BMDCs and BMDMs**

To determine whether rat BMDCs and BMDMs mature and respond appropriately to inflammatory stimuli, rat cells were exposed to a panel of TLR ligands or media alone for 24 h. LPS and poly I:C, but not R837, stimulation significantly increased the percentage of DCs expressing MHCII, CD80, and CD86, production of TNF-α and IL-6, and expression of Ifnβ compared with cells exposed to media alone (P<0.05 for each; Fig. 1). Concentrations of IL-10 and TGF-β were not altered by any TLR ligand treatment.

Compared with media alone, stimulation with LPS, poly I:C, and R837 significantly increased the percentage of BMDMs expressing MHCII and CD80, but not CD86 (P<0.05 for each; Figs. 2A and B). The production of IL-6 and IL-10 and the expression of Ifnβ were significantly enhanced in macrophages stimulated with LPS, poly I:C, and R837, whereas TNF-α production was increased only in response to LPS and poly I:C (P<0.05 for each; Figs. 2C and D). Production of TGF-β was not changed in macrophages following stimulation with any of the TLR ligands. Taken together, these data indicate that DCs and macrophages differentiated from rat BM respond to diverse TLR ligands with vigorous but distinct inflammatory responses.

**SEOV infection does not induce maturational changes in rat BMDCs and BMDMs**

In order to determine the maturational changes in APCs during SEOV infection, BMDCs and BMDMs were infected with SEOV at various MOIs and compared with APCs stimulated with poly I:C or media alone. Negative cell, cell-associated SEOV RNA was detectable at low copy numbers 1 day p.i. in BMDCs and BMDMs and increased over time with the highest copy numbers detected 6 days p.i. in cells infected at the highest MOI (P<0.05 for each; Figs. 3A and B). Although SEOV RNA was detected in both BMDCs and BMDMs, the quantity of negative sense viral RNA was 10-fold higher in BMDMs than in BMDCs (P<0.05). Positive sense, cell-associated SEOV RNA was detected in BMDCs infected with a MOI of 5 at 6 days p.i., in BMDMs at 4 and 6 days p.i. in the cells infected with a MOI of 5, and at 6 days p.i. in BMDMs infected with SEOV at a MOI of 0.5 (data not shown). SEOV N protein was detectable in both BMDCs and BMDMs at 6 days p.i., but the number of SEOV antigen-positive cells was higher in BMDMs than in BMDCs (Figs. 3C and D). No viral antigen was detected in mock-infected BMDCs or BMDMs (data not shown).

Infectious virus particles were not detected in the supernatant of BMDCs infected with SEOV at 6 days p.i. (data not shown). Low levels of infectious virus particles were detected in the supernatant of BMDMs, with the highest amount of infectious SEOV detected in the supernatant of cells infected with a MOI of 5 (P<0.05; Fig. 3E). Copies of negative sense SEOV RNA from infected cells were positively correlated with infectious SEOV TCID50 units, with one copy of SEOV RNA = 0.25 TCID50 units (P<0.05).

The percentage of BMDCs and BMDMs expressing MHCII, CD80, and CD86 was increased in cells stimulated with poly I:C, but not in APCs infected with SEOV (Figs. 4A–D). Production of TNF-α, IL-6, and IL-10 (Figs. 4E–I, K) as well as the expression of Ifnβ (Figs. 4M and N) were significantly elevated in DCs and macrophages stimulated with poly I:C, but not in mock or SEOV-infected DCs or macrophages (P<0.05 in each case). In contrast, concentrations of TGF-β were significantly reduced in BMDCs and BMDMs infected with SEOV as compared with either poly I:C-stimulated or mock-infected cells (P<0.05 for each; Figs. 4J and L). The viability of BMDCs and BMDMs was not differentially affected by the treatment of cells with SEOV, poly I:C, or media alone at any of the time points examined (data not shown). Taken together, these results suggest that SEOV infection of APCs does not activate the expression of surface markers or cytokine production as is evident with poly I:C treatment.

**Pretreatment of BMDCs and BMDMs with poly I:C suppresses SEOV replication**

To determine whether the environment in which DCs and macrophages encounter SEOV could alter cellular responses to infection as
well as SEOV replication, cells were pretreated with poly I:C for 24 h, infected with SEOV (MOI of 5), washed, and monitored for viral RNA synthesis. Pretreatment with the TLR3 ligand, poly I:C prior to infection with SEOV significantly reduced the amount of SEOV RNA 6 days p.i. in BMDCs and BMDMs as compared with cells infected with SEOV alone (Pb0.05 in each case, Figs. 5A and B). BMDCs and BMDMs treated with poly I:C prior to SEOV infection transiently expressed more MHCII on their surface on day 1 p.i. and CD80 on their surface 1–2 days p.i. than did cells infected with SEOV alone (P<0.05 in each case, Figs. 5A and B).

BMDCs and BMDMs treated with poly I:C prior to SEOV infection transiently expressed more MHCII on their surface on day 1 p.i. and CD80 on their surface 1–2 days p.i. than did cells infected with SEOV alone (P<0.05 in each case; Figs. 6A–D). In DCs, pretreatment with poly I:C prior to infection significantly increased the production of TNF-α, but not IL-6 or IL-10, or the expression of Ifn-β, as compared with DCs infected with SEOV alone (P<0.05, Figs. 6E, F, I, and M). In contrast, activation of macrophages with poly I:C prior to SEOV infection significantly increased the production of IL-6, IL-10, and TNF-α 1–6 days p.i. as well as the expression of Ifn-β 1 day p.i. as compared with cells infected with SEOV alone (P<0.05 in each case; Figs. 6G, H, K, and N). Concentrations of TGF-β were not affected by treatment of cells with poly I:C before infection; APCs infected with SEOV produced less TGF-β than cells treated with either poly I:C or media alone (P<0.05 in each case, Figs. 6J, K, L, and M). The viability of DCs and macrophages was not affected by either SEOV infection or pretreatment of cells with poly I:C at any of the time points examined (data not shown). Taken together, these data illustrate that establishment of an antiviral state in DCs and to a greater extent in macrophages can increase cytokine production and reduce replication of SEOV in APCs from the natural host.

SEOV suppresses LPS-induced inflammatory responses in APCs

To determine whether SEOV suppresses the functioning of APCs in response to an immune stimulus, DCs and macrophages were infected with SEOV (MOI of 5) and subsequently activated with the TLR4 ligand, LPS at several time points p.i. LPS was selected for this series of experiments, because it has been used previously to demonstrate virus-induced suppression of innate immune cell activation (Lore...
et al., 1999; Beck et al., 2003; Yao et al., 2007). Negative-sense, cell-associated SEOV RNA was detectable at low copy numbers at 1 day p.i., and the amount of viral RNA increased significantly by 6 days p.i. in all cells infected with SEOV (P < 0.05 for each; data not shown). Positive-sense, cell-associated RNA was only detectable at 4 and 6 days p.i. in cells infected with SEOV (data not shown).

Infection of BMDCs and BMDMs with SEOV prior to stimulation with LPS had no effect on cell viability (data not shown) but reduced the surface expression of MHCII and CD80 (P < 0.05 for each; data not shown), but not CD86 (data not shown), as compared with cells stimulated with LPS alone, at all time points examined. Infection of DCs and macrophages with SEOV also suppressed LPS-induced production of TNF-α, IL-6, and IL-10 (Figs. 7E–H, I, and K), and the expression of Ifn-β (Figs. 7M and N; P < 0.05 for each) but did not affect concentrations of TGF-β, which were suppressed in all SEOV-infected cells as compared with mock-infected cells and cells stimulated with LPS alone (P < 0.05 for each; Figs. 7J and L).

NF-κB is a transcriptional factor that activates the transcription of many proinflammatory and antiviral genes. To determine whether SEOV suppresses inflammatory responses by mitigating activity along the NF-κB signaling pathway, PCR arrays were used to compare the expression of NF-κB-focused genes in BMDMs that were mock infected, stimulated with LPS, infected with SEOV, or infected with SEOV prior to stimulation with LPS and collected 24 h after treatment. Macrophages were selected for these analyses because they support significantly more SEOV replication than DCs. When compared with mock-treated cells, incubation of macrophages with LPS alone significantly upregulated the expression of 40/84 genes (47.6% with N2-fold, P < 0.05 in each case; Supplemental Table 1), whereas SEOV infection alone did not significantly alter the expression of any NF-κB-related genes as compared with mock-treated BMDMs. Infection of macrophages with SEOV prior to stimulation with LPS led to upregulation of 40/84 genes and downregulation of 2/84 genes (2.4%) as compared with mock-treated cells (N2-fold, P < 0.05 in each case).

Fig. 2. TLR ligands induce inflammatory responses in macrophages from Norway rats. BMDMs were cultured for 8 days and stimulated with LPS (1 μg/ml), poly I:C (1 μg/ml), or R837 (1 μg/ml) for the last 24 h of culture. (A) Representative histograms of adherent cells harvested and stained for FACS analysis are shown. Gray line: isotype control; shaded histogram: untreated BMDMs; solid line: TLR stimulated BMDMs. (B) Values represent either the percentage of total cells (CD163+) or the percentage of CD163+ BMDMs expressing MHCII, CD80, and CD86 following stimulation with the indicated TLR ligands. (C) Culture supernatants were collected after 24 h of stimulation with TLR ligands and examined for mean production of TNF-α, IL-6, IL-10, and TGF-β by ELISA. (D) Expression of Ifn-β in BMDMs was measured at day 8 by real-time RT-PCR and is normalized to Gapdh and the expression levels of the media control. Results are representative of three independent experiments. The vertical bars indicate means ± SEM and an asterisk (*) indicates that the TLR ligand enhanced responses compared with media alone. P < 0.05.
Infection of macrophages with SEOV prior to stimulation with LPS significantly downregulated the expression of Casp8, Cd40 (Tnfsf5), Ifta, Ikbke, II10, Iff1, Iff3, Iff1, Iff1, Stat1, Thr1, Thr9, Tnfsf10, and Zap70 and upregulated the expression of Cs32, Cs33, Ifta1, Ifta, and Iffb as compared with treatment of BMDMs with LPS alone (>2-fold, \( P < 0.05 \) in each case; Fig. 8). These data indicate that although SEOV infection alone causes limited changes in NF-κB-related gene expression, infection can significantly alter NF-κB signaling in response to LPS; thus, SEOV infection dampens macrophage responses to an inflammatory stimulus.

**Discussion**

Rodent reservoirs infected with their species-specific hantaviruses develop long-term, persistent infections, shedding virus intermittently in urine, feces, and saliva (Easterbrook and Klein, 2008a).
Heightened activity of regulatory T cells in combination with reduced antiviral and proinflammatory responses underlies the persistence of hantaviruses in rodent reservoirs (Easterbrook et al., 2007; Schountz et al., 2007; Easterbrook and Klein, 2008b, 2008c). The cellular mechanisms mediating induction of regulatory responses in rodents have not been evaluated for any hantavirus-rodent system. The primary goal of the present study was to establish whether SEOV alters the functioning of APCs from its natural host. We demonstrate that although APCs derived from rats can respond to inflammatory stimuli (e.g., TLR ligands) with appropriate proinflammatory responses, SEOV infection fails to induce the expression of activation markers, including MHCII and CD80, and production of appropriate antiviral and proinflammatory responses in DCs and macrophages. These responses were evident in cells pre-treated with poly I:C prior to SEOV infection. SEOV also suppresses the ability of APCs to respond to subsequent TLR ligand stimulation.

Several viruses that cause persistent infections, including human cytomegalovirus (HCMV), lymphocytic choriomeningitis virus (LCMV), human immunodeficiency virus (HIV), hepatitis C virus (HCV), and herpes simplex virus (HSV) reportedly infect DCs and inhibit maturation and T cell stimulatory capacity (Lore et al., 1999; Salio et al., 1999; Beck et al., 2003; Triullo et al., 2006). Our data support and extend these findings by illustrating that a zoonotic pathogen like SEOV can modulate the maturation and functioning of both DCs and macrophages which may be a fundamental mechanism leading to persistence of zoonotic pathogens in reservoir hosts. The suppressive effects of SEOV on rat APC activity is in contrast with the increased activity of human DCs (Raftery et al., 2002) and macrophages (Temonen et al., 1995; Cebalo and Markotic, 2007) following infection with hantaviruses. We hypothesize that the divergent effects of hantavirus infection on APCs from rats as compared with humans may be critical in explaining the differential outcome of hantavirus infection in humans and rodent reservoirs.

We have shown previously that SEOV infects both macrophages and endothelial cells in the lungs of rats (Easterbrook and Klein, 2008b). Although SEOV infected both DCs and macrophages from rats,

![Image](38x314 to 549x741)

**Fig. 4.** SEOV does not induce inflammatory or antiviral responses in DCs or macrophages from Norway rats. After 7 days of differentiation, BMDCs and BMDMs were inoculated with media alone or media containing SEOV at a MOI of 0.05, 0.5, or 5. At 1, 2, 4, and 6 days p.i., non-adherent DCs and adherent macrophages were harvested for FACS analysis. Values represent the percentage of CD11c+ BMDCs and CD163+ BMDMs expressing MHCII and CD80 following stimulation (A–D). Culture supernatants were collected 1, 2, 4, and 6 days p.i. and examined for mean production of TNF-α, IL-6, IL-10, and TGF-β by ELISA (E–L). Expression of Ifnβ in BMDCs and BMDMs was measured at the indicated times by real-time RT-PCR and is normalized to Gapdh and the expression levels of mock-infected cells (M and N). Results are representative of three independent experiments. The values indicate means ± SEM.
and replication, affect the innate immune responses of infected cells. To examine how the recognition of hantavirus-associated molecular patterns by pattern recognition receptors, as opposed to virus entry and replication, affect the innate immune responses of infected cells.

Induction of type I IFNs and proinflammatory cytokines are critical components of the innate immune response to viruses. Infection of human endothelial cells with hantaviruses that cause known disease in humans (e.g., HTNV, SEOV, SNV, New York-1 virus [NY-1V], Puumala virus, and Andes virus) results in delayed induction of type I IFNs and ISGs as compared with cells infected with hantaviruses that cause either no known or mild disease in humans (e.g., Tula virus and Prospect Hill virus) (Geimonen et al., 2002; Kraus et al., 2004; Khaiboullina et al., 2005; Spiropoulou et al., 2007). Recent studies further illustrate that induction of type I IFNs, ISGs, and proinflammatory cytokines requires TLR3, and possibly TLR4, in human endothelial cells (Jiang et al., 2008; Handke et al., 2009). Exposure of APCs from rats to the TLR3 ligand poly I:C prior to SEOV infection significantly increased the expression of activation markers, production of proinflammatory cytokines, and expression of Ifnβ. Although pretreatment of cells with poly I:C prior to SEOV infection increased activation and cytokine production by DCs and macrophages, levels still were below those seen in cells treated with poly I:C alone, suggesting SEOV infection is still damping the responses of these cells. The impact of poly I:C on antiviral and proinflammatory responses was more pronounced for macrophages than for DCs. Importantly, generation of an antiviral state via TLR stimulation prior to infection significantly reduced the amount of SEOV RNA in both macrophages and DCs, illustrating that the suppressive effects of SEOV on innate immune responses are dependent on the environment in which APCs encounter virus. Furthermore, antiviral and proinflammatory cytokines may promote activation of APCs and reduce persistence of hantaviruses in rodent reservoirs.

When SEOV-infected DCs and macrophages were stimulated with LPS, the synthesis of proinflammatory cytokines and the expression of Ifnβ were suppressed as compared with cells stimulated with LPS alone. The mechanism of hantavirus-induced suppression of inflammatory responses in rodents may involve downregulation of the NF-κB and interferon regulatory factor (IRF) signaling pathways. Interference with these signaling pathways by hantaviruses has been reported. The HTNV N protein, for example, is able to sequester NF-κB in the cytoplasm, thereby inhibiting NF-κB activity and the subsequent downstream effector responses in human kidney and lung epithelial cells (Taylor et al., 2009). The cytoplasmic G(0) tail of NY-1V prevents phosphorylation and nuclear translocation of IRF-3 and NF-κB, thereby inhibiting IFN transcription (Alff et al., 2008).

To begin to address how SEOV suppresses antiviral and proinflammatory responses in APCs, the expression of genes along the NF-κB signaling pathway was examined in macrophages. NF-κB is a key mediator involved in the initiation of the innate immune response. In the present study, LPS was a potent inducer of the classical, canonical NF-κB signaling pathway in macrophages from rats. In contrast, SEOV infection did not induce any genes associated with NF-κB signaling in macrophages. Further, infection of cells with SEOV prior to activation with LPS significantly reduced the expression of several clusters of genes involved in NF-κB signaling. TLRs and RNA helicases require caspase-8 for activation of NF-κB (Maelfait and Beyaert, 2008) and SEOV disrupted the activity along this pathway by suppressing the expression of several TLRs (e.g., Tlr1 and Tlr9) and Casp8. Ifnβ is another transcriptional factor that was suppressed in macrophages infected with SEOV prior to stimulation with LPS. Interferon-γ is the strongest inducer of Ifnβ, which can interact with both Stat1 and NF-κB to induce the synthesis of proinflammatory products (Kroger et al., 2002). Ifnβ also transcriptionally regulates Ifnβ. SEOV significantly suppressed the LPS-induced expression of Ifng, Ifnl, Stat1, and Ifnβ. Proinflammatory cytokines, including IFN-γ, are regulated by IL-10. In the present study and in our previous in vivo studies (Easterbrook et al., 2007; Easterbrook and Klein, 2008b), Il10 expression was suppressed by SEOV suggesting that the regulatory balance of IFN-γ and IL-10 is disrupted by SEOV infection. The expression of genes of the TNF receptor super family, including Lta, Trifβ10, and Trifβ5 (i.e., Cld40), which modulate inflammatory responses that are transcriptionally regulated by NF-κB (Schneider et al., 2004), also was suppressed by infection with SEOV prior to LPS stimulation. These genomic pathways provide data to form testable hypotheses about the molecular mechanisms mediating SEOV inhibition of rat APC functions in response to an immune stimulus. To further evaluate the functional significance of these data, future studies will address
whether SEOV suppresses NF-κB signaling in alveolar macrophages in rats.

We initially hypothesized that SEOV may induce a regulatory phenotype in APCs from rats because in vivo studies have demonstrated that elevated production of TGF-β precedes the increased number of regulatory T cells in the lungs (Easterbrook et al., 2007; Schountz et al., 2007; Easterbrook and Klein, 2008b). In the present study, production of both IL-10 and TGF-β in DCs and macrophages was suppressed by SEOV, suggesting that these cells do not contribute to the elevated TGF-β concentrations observed in infected rodents as early as 72 h after infection (Schountz et al., 2007; Easterbrook and Klein, 2008b). Many cell types, including lymphocytes, endothelial cells, epithelial cells, and smooth muscle cells, can produce TGF-β; thus, the heightened regulatory response observed in vivo may be a consequence of cytokine secretion from multiple cell types at the site of infection (Kehrl et al., 1986; Pintavorn and Ballermann, 1997; Li and Flavell, 2008). The vascular endothelium is a primary target of hantavirus infection and many studies have utilized human endothelial cell lines to evaluate the immune response to infection (Yanagihara and Silverman, 1990; Pensiero et al., 1992; Zaki et al., 1995; Sundstrom et al., 2001). Although we have demonstrated that lung endothelial cells are infected with SEOV in vivo (Easterbrook and Klein, 2008b), no studies have evaluated the effect of hantavirus infection on the functioning of rat endothelial cells, which may both support elevated virus replication and produce significant amounts of TGF-β during infection.

The data presented provide novel information about the impact of a zoonotic pathogen on the function of innate immune cells in a reservoir host. By suppressing antiviral and proinflammatory defenses of innate immune cells and upregulating the activity of regulatory T cells in rats, SEOV is able to cause a persistent infection. These data provide insight into the mechanisms by which zoonotic viruses have
remained in the environment for millions of years and continue to be transmitted to humans.

**Materials and methods**

**Animals**

Adult male (60–70 days of age) Lewis rats were purchased from Charles River Laboratories (Raleigh, NC) and maintained in pathogen-free facilities with a constant 14:10 light/dark cycle. All procedures were performed in accordance with the Johns Hopkins Animal Care and Use Committee (Protocol No. RA07H190) and the Johns Hopkins Office of Health, Safety and Environment (Protocol No. P9902030110).

**Generation of bone marrow-derived DCs and macrophages**

Bone marrow cells were isolated from femurs and tibiae of adult male rats and cultured (1 × 10^6 cells/ml) in RPMI media (Mediatech, Inc., Manassas, VA) supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 50 μM 2-β-mercaptoethanol (Invitrogen and Sigma). To differentiate cells into BMDCs, media was further supplemented with rGM-CSF (25 ng/ml; Peprotech, Rocky Hill, NJ) and rIL-4 (5 ng/ml; Peprotech). Differentiation of BM cells into macrophages was achieved by culturing cells in medium supplemented with 30% culture supernatant from L929 mouse fibroblast cells, as previously described (Boltz-Nitulescu et al., 1987). On days 3 and 6, half of the medium was aspirated and replaced with fresh medium containing growth factors.

**Virus**

SEOV (strain SR-11; 1.5 × 10^7 plaque forming units (PFU)/ml) was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Ft. Detrick, MD), wherein virus was isolated from neonatal rat brains and passaged in Vero E6 cells in EMEM supplemented with 10% FBS as described previously (Boltz-Nitulescu et al., 1987). On days 3 and 6, half of the medium was aspirated and replaced with fresh medium containing growth factors.

**Stimulation and infection of bone marrow cell cultures**

For stimulation of cells with TLR ligands, cells were treated with the TLR4 ligand LPS (1 μg/ml; Sigma-Aldrich, St. Louis, MO), the TLR3 ligand poly I:C (1 μg/ml; Invivogen, San Diego, CA), or the TLR7 ligand 4-

![Fig. 7. SEOV suppresses LPS-induced activation of DCs and macrophages. After 7 days of differentiation, BMDCs and BMDMs were inoculated with media alone or media containing SEOV (MOI of 5). Mock and SEOV-infected cells were stimulated with LPS (1 μg/ml) 24 h prior to harvesting cells. At 1, 2, 4, and 6 days p.i., non-adherent DCs and adherent macrophages were harvested for FACS analysis and values represent the percentage of CD11c+ BMDCs and CD163+ BMDMs expressing MHCII and CD80 following SEOV infection (A–D). Culture supernatants were collected at the indicated times and analyzed for mean production of TNF-α, IL-6, IL-10, and TGF-β by ELISA (E–L). Expression of Ifnβ in BMDCs and BMDMs was measured at 1, 2, 4, and 6 days p.i. by real-time RT-PCR and is normalized to Gapdh and the expression levels of mock-infected cells (M and N). Results are representative of three independent experiments. The values indicate means ± SEM.](image-url)
R837 (1 μg/ml; Invivogen) for 24 h. For virus infection, cells were exposed to DMEM supplemented with 2% FBS alone or media containing specified concentrations of SEOV for 2 h at 37 °C. For BMDCs, cells (1 × 10^6 cells/well) were harvested from three wells, centrifuged, and pelleted for infection. For BMDMs, 1 × 10^6 cells/well were infected for the specified amount of time. After 2 h, cells were washed and incubated at 37 °C for 1, 2, 4, or 6 days post infection (p.i.) at the indicated multiplicities of infection (MOI) in RPMI supplemented with 10% FBS, antibiotics, and cytokines. For studies in which APCs were treated with poly I:C prior to infection, cells were exposed to poly I:C (1 μg/ml) for 24 h, infected with SEOV (MOI of 5) for 2 h, washed, and incubated for the specified number of days p.i. For studies in which APCs were infected with SEOV and subsequently stimulated with LPS, cells were infected (MOI of 5) for 2 h, washed, and then stimulated with LPS (1 μg/ml) for 24 h prior to harvesting at the specified time points p.i. For each experiment, viable cells were counted using a hemacytometer and trypan blue exclusion. All virus infections were performed under university-approved BSL-3 protocols.

**Flow cytometric analyses**

Cultured cells were collected, washed in FACS buffer containing PBS and 5% FBS, and counted. Nonspecific binding was blocked with FcR (Invitrogen, Carlsbad, CA). Cells were incubated with the

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**Fig. 8.** SEOV suppresses NF-κB signaling activity following stimulation of macrophages with LPS. The rat NF-κB signaling pathway RT2 Profiler PCR Array was used to examine the expression of 84 pathway-focused genes and the resulting gene expression values were analyzed by one-way ANOVA and only genes that demonstrated a >2-fold change with \( P < 0.05 \) in the contrast comparison of expression values from experimental treatment cells and mock-infected cells were considered differentially expressed. Represented in the custom pathway is the fold change in the expression of genes from BMDMs infected with SEOV and subsequently stimulated with LPS relative to either mock-treated or LPS-treated cells. The contrast comparisons were imported into IPA and clustered into functional groups based on biological function to create a network of genes that are associated with NF-κB-mediated inflammatory responses and that are differentially expressed following SEOV infection. Gray lines indicate direct relationships between genes. See Supplemental Table 1 for the complete list of genes and fold change values.

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**Contrast Comparison Key:**
- 3+ fold > mock-treated
- 3+ fold > mock-treated, but 3+ fold < LPS-treated
- 3+ fold < LPS-treated, but n.s. change from mock-treated
- n.s. change from mock-treated and LPS-treated
following panel of mouse anti-rat antibodies: CD11c-A647 (clone 8A2; AbD Serotec, Raleigh, NC), CD163-A647 (clone ED2; AbD Serotec), MHCIIFITC (clone OX6; AbD Serotec), CD80-APC (clone 3H5; Invitrogen), and CD86-FITC (clone 24F; Invitrogen). Cells were fixed in 2% paraformaldehyde for 30 min which is necessary for inactivating virus for processing outside of a BSL-3 (Kraus et al., 2005). Cells were sorted using the CellQuest Pro software and the BD FACSCalibur machine (BD Biosciences). Data were analyzed using the FlowJo analysis software (Treestar, Inc., Ashland, OR).

ELISAs for cytokine quantification

For experiments involving exposure to viruses, supernatant samples were exposed to UV irradiation for 3 min to inactivate virus for processing of samples outside of a BSL-3. Pilot experiments confirmed the efficacy of UV irradiation at eliminating infectious virus without altering the ability to detect cytokines in these samples. Cytokine protein in cell supernatants was measured using ELISA kits for rat IL-6, IL-10, TNF-α, and mouse TGF-β (R&D Systems, Minneapolis, MN) following the manufacturer’s protocol.

RNA isolation

RNA was isolated from cultured cells using Trizol LS (Invitrogen) following the manufacturer’s protocol as previously described (Hannah et al., 2008). RNA concentration and purity was measured using a NanoDrop (ThermoFisher Scientific, Waltham, MA).

Real-time RT-PCR for host gene expression

First strand cDNA was prepared from 1 μg RNA isolated from cultured cells, 2.5 μM oligo(dT)20 primer and the manufacturer’s protocol for Superscript III First Strand Synthesis (Invitrogen). Custom primer and probe sets were generated for rat Gapdh and Ifng using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). cDNA was amplified in duplicate in a reaction mixture containing TaqMan Universal Master Mix, 20 μM of each primer, and 10 μM of probe, following the manufacturer’s protocol for final concentrations and amplification profile. All reactions were performed in 96-well optical reaction plates using the ABI 7300 Sequence Detection System (Applied Biosystems). Serial dilutions of pooled cDNA from selected samples were utilized to generate a standard curve, which was run on each plate. Gene expression patterns were normalized to Gapdh expression and are presented as relative to the expression levels of media control cells.

Real-time RT-PCR for SEOV S segment

Synthesis of the S segment of SEOV cDNA was prepared for the negative and positive strand using 0.1 μM gene-specific primers and the reagents from the Superscript III First Strand Synthesis kit following the manufacturer’s protocol (Invitrogen) as described previously (Hannah et al., 2008). Amplification of an 81-bp nucleotide sequence of the negative strand and the 60-bp positive strand of viral RNA was achieved in the same reaction mixture as described previously (Botten et al., 2003; Hannah et al., 2008). Positive and negative controls as well as a standard curve ranging from 10 to 10^6 copies of SR-11 S segment in plasmid pWRG7077 were run on each plate. All reactions were multiplexed in 96-well optical plates using the ABI 7300 Sequence Detection System (Applied Biosystems).

PCR array for gene expression profiling of the NF-κB signaling pathway

The rat NF-κB signaling pathway RT² Profiler PCR Array was utilized to examine 84 genes associated with NF-κB-mediated signal transduction, housekeeping genes, and RNA and PCR quality controls according to the manufacturer’s protocol (SA Biosciences, Frederick, MD). Complementary DNA was prepared from 500 ng of RNA using the RT² First Strand kit and amplified in a total volume of 25 μl in a reaction mixture containing RT² SYBR Green/ROX PCR Master Mix (SA Biosciences) in the ABI 7300 Sequence Detection System (Applied Biosystems). The threshold cycle (Ct) was calculated for each well, the Ct values of the control wells were confirmed, and values for the 84 pathway-focused genes were analyzed using a modified version of the ∆∆Ct method. First, to calculate ∆Ct, the Ct for the housekeeping gene Rplp1 (i.e., the housekeeping gene that yielded the most consistent results across all PCR array plates and conditions) was subtracted from the Ct for each pathway-focused gene. The resulting value was multiplied by −1 to account for the inverse proportional relationship between the Ct and the calculated gene expression value. To determine the fold change in gene expression following treatment (∆∆Ct), ∆Ct values from mock infected treatment samples were subtracted from ∆Ct values for genes from experimental cells. The resulting gene expression values were analyzed by a one-way ANOVA using Partek Genomics Suite v. 6.4 (Partek Incorporated, St. Louis, MO) and only genes that demonstrated a >2-fold change with P < 0.05 in the contrast comparison of expression values from experimental treatment cells and mock infected cells were considered significantly differentially expressed. The contrast comparisons were imported into Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc, Redwood City, CA) for visualization.

Immunohistochemistry

At 6 days p.i., mock and SEOV-infected (MOI of 5) cells were washed with PBS, fixed in 2% paraformaldehyde, permeabilized with PBS containing 0.2% Triton X-100 and 0.1% sodium citrate, and blocked in buffer containing PBS, 3% normal goat serum, and 0.5% BSA. For BMDCs, cytocentrifuge preparations were made using the Shandon Cytospin 3 (ThermoScientific, Waltham, MA). Cells were incubated with primary rat anti-ANDV N antibody (which was determined to be cross-reactive with SEOV during pilot experiments) diluted in blocking buffer (1:1500), washed, and incubated with secondary antibody (goat anti-rat Alexa Fluor 488 diluted 1:500; Molecular Probes, Carlsbad, CA) (Rowe et al., 2008). Cells were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA). Fluorescence emission images were visualized (Eclipse E800, Nikon Inc., Melville, NY) and merged using a SPOT charge-coupled device camera and software (Diagnostic Instruments, Sterling Heights, MI) at a magnification of 40×.

TCID₅₀ assay

SEOV-infected cell supernatants were diluted in serial 10-fold dilutions in DMEM (Invitrogen) containing 2% FBS. Vero E6 cells were grown in 96-well plates until confluent and then infected with 100 μl of cell supernatant per well in sextuplicate. SEOV-infected Vero E6 cells were incubated at 37 °C with 5% CO₂ for 12 days, washed with PBS, and permeabilized and fixed with a solution of cold 95% ethanol/5% acetic acid. For immunostaining, cells were incubated in blocking buffer containing 3% BSA (Sigma-Aldrich) in PBS followed by incubation with rat anti-ANDV N antibody diluted in blocking buffer (1:5000). Cells were incubated with goat anti-rat horseradish peroxidase (HRP) substrate (1:5000; Jackson Immunoresearch, Westgrove, PA), washed, and nucleocapsid-positive wells were visualized after the addition of TMB substrate (BD Biosciences) as described previously (Rowe et al., 2008). Virus titers were calculated using the Reed-Muench formula.

Statistical analyses

Copies of SEOV RNA, infectious virus particles, cell viability, host gene expression, surface marker expression, and protein concentra-
tions were analyzed by either parametric or non-parametric ANOVAs depending on the normality of the distribution with two between group variables (treatment and day p.i.). Significant interactions were further analyzed using the Tukey or Dunn method for pairwise multiple comparisons. Mean differences were considered statistically significant if $P<0.05$.

Disclosures
The authors have no financial conflict of interest.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.01.027.

References


