Cytokine response in mouse bone marrow derived macrophages after infection with pathogenic and non-pathogenic Rift Valley fever virus

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INTRODUCTION

Rift Valley fever virus (RVFV) (family Bunyaviridae, genus Phlebovirus) is a pathogenic mosquito-borne virus known to cause lethal disease in humans and ruminants (Al Azraqi et al., 2013; Davies, 2010; Findlay & Daubney, 1931; Mapaco et al., 2012; Schwentker & Rivers, 1934). In humans, the clinical picture can vary from a self-limiting febrile disease, to severe symptoms, such as haemorrhagic fever, encephalitis and retinitis, and can ultimately result in death (Abdel-Wahab et al., 1978; Gear, 1977; Maar et al., 1979). RVFV is endemic to sub-Saharan Africa, Egypt and the Arabian Peninsula (Bird et al., 2009) and is primarily transmitted by the Aedes mcintoshi mosquito (Swanepoel & Coetzter, 1994). Aedes spp. of mosquitoes have a worldwide distribution, providing the possibility for introduction of RVFV into new areas (Davies & Martin, 2003; Kasari et al., 2008; Swanepoel & Coetzter, 2004).

Much effort has been made toward development and evaluation of antivirals and vaccines, but detailed information regarding the molecular mechanisms directing host responses against RVFV infection is still insufficient.
A critical component of the host response to RVFV infection is a robust type I IFN response shortly after infection (do Valle et al., 2010). Previous work has shown that RVFV can evade the host immune response through manipulation of type I IFN responses (Bouloy et al., 2001) and the NSs protein is a critical component of RVFV type I IFN antagonism (Billecocq et al., 2004; Ermler et al., 2014; McElroy & Nichol, 2012). The importance of type I IFN and interferon-stimulated genes (ISGs) during RVFV infection has also been demonstrated in MBT/Pas mice (do Valle et al., 2010). Even though studies have shown that NSs blocks the transcription of type I IFN (Bouloy et al., 2001), it appears that injection of MBT/Pas mice with pathogenic RVFV does lead to small amounts of IFN secretion (do Valle et al., 2010; Le May et al., 2008). do Valle et al. (2010) suggested that the slightly stronger IFN response in BALB/cByJ mice is one of the factors contributing to longer survival after infection with RVFV when compared with the MBT/Pas mice. Endogenous IFN levels contribute to host survival even in the presence of RVFV mediated inhibition of ISG transcription (Bouloy et al., 2001; Le May et al., 2008; Morrill et al., 1989, 1990).

McElroy & Nichol (2012) demonstrated that infection with a pathogenic wild type (WT) RVFV strain ZH501 did not induce type I IFN (IFN-α2 and IFN-β) or TNF-α production in primary human monocyte derived macrophages (MDMs), while infection with a recombinant RVFV lacking NSs resulted in increased IFN-α2, IFN-β and TNF-α. Ermler et al. (2014) showed that NSs deletion mutants cause mouse bone marrow derived macrophages (BMDM) to produce greater amounts of IFN-α when compared with the attenuated vaccine strain MP-12, which encodes an intact NSs protein. Narayanan et al. (2011) found that infection with RVFV vaccine strain MP-12 or pathogenic strain ZH501 resulted in increased TNF-α in primary human small airway lung epithelial cells. These data support the notion of cell type specific responses to RVFV infection.

Studies evaluating the effect of IFN-γ following WT RVFV infection found that IFN-γ release in infected mice had little impact on virus propagation (Jansen van Vuren et al., 2011). We also found there was little to no change in IFN-γ secretion after infection of C57BL/6 mice with either MP-12 or ZH501 (Gray et al., 2012). IFN-γ was shown to play an important role during RVFV infection in rhesus macaques. Macaques given IFN-γ replacement therapy were able to control viral infection, demonstrated by a decrease in viraemia and protection from fatal disease when compared with control macaques (Morrill et al., 1990, 1991).

Macrophages are phagocytic antigen presenting cells that are found in all tissues. During viral infection, macrophages release cytokines and chemokines that not only stimulate and recruit cells of the innate immune system, but also activate the adaptive immune system. Studies using Chikungunya and human immunodeficiency virus as well as haemorrhagic fever viruses such as Ebola Zaire and RVFV demonstrated that viruses can replicate in macrophages (Bol et al., 2011a, 2011b; Bray & Geisbert, 2005; Dupuis-Maguiraga et al., 2012; Lewis et al., 1987; McElroy & Nichol, 2012). Kupffer cells, resident liver macrophages, as well as circulating white blood cells have been shown to stain positive for RVFV antigen after infection (Kamal, 2009; Shieh et al., 2010; Smith et al., 2010) and it is thought that macrophages could support dissemination of the virus from the site of infection to target organs as has been shown with Ebola virus (Bray & Geisbert, 2005). Here, we wanted to characterize the response of primary mouse BMDM cells to infection with either WT or attenuated strains of RVFV and correlate cytokine profiles with regulation of intracellular signalling processes. These responses of antigen presenting immune cells likely contribute to the host response to RVFV infection with potentially important implications for disease development. Furthermore, we performed a comparative study in the widely used mouse macrophage cell line RAW 264.7, to evaluate the suitability of using immortalized murine cell lines in studies characterizing the cellular response to RVFV infection. Similar to results published by McElroy & Nichol (2012) for human MDMs, we detected an increased secretion of T helper (Th) 1-associated antiviral cytokines and chemokines in primary murine BMDMs after infection with a recombinant RVFV lacking 70 % of the NSs protein (rMP12-C13 type virus) (Ikegami et al., 2006), when compared to attenuated MP-12 or WT ZH501. While cytokine responses after MP-12 infection appeared delayed and less intense, cytokine responses to ZH501 infection appeared to be largely absent when compared to rMP12-C13 type infected BMDMs.

RESULTS

RVFV replication in immortalized and primary mouse macrophages

Multi-step growth kinetics for RVFV ZH501, MP-12 and rMP12-C13 type were determined in BMDM, as well as RAW 264.7 cells (Fig. 1). In primary BMDM, ZH501 grew to a titre of 5 log_{10} p.f.u. ml^{-1} at 24 h post-infection (p.i.), while MP-12 and rMP12-C13 type reached a peak titre of just under 4 log_{10} p.f.u. ml^{-1} (Fig. 1a). Beyond 24 h, the viral titres gradually decreased until termination of the experiment at 72 h p.i. At 72 h p.i., approximately 90 % cytokine effect (CPE) was visually detected (rounded cells) in ZH501 infected cells, and approximately 20 % CPE in MP-12 infected cells. No obvious cell death was observed after infection with rMP12-C13 type virus (data not shown). An initial dip in viral titre between 1 and 6 h p.i. was detectable, likely caused by uptake of residual virus into BMDM. In contrast, MP-12, rMP12-C13 type and ZH501 infection of RAW 264.7 cells reached peak titres of about 6 log_{10} p.f.u. ml^{-1} within 12–24 h p.i. (Fig. 1b). While there was no CPE observed after rMP12-C13 type infection in RAW 264.7 cells, CPE began at 18 h p.i. in MP-12 infected cells and reached approximately 50 % at 72 h p.i. In ZH501 infected cells, CPE was seen 12 h p.i. and reached about 95 % cell death by 24 h p.i.
Cytokine response in primary mouse BMDMs

To further define the response of BMDMs to infection with WT, attenuated or NSs deficient RVFV, we first examined expression of IFN-β following infection (Fig. 2). An increase in secreted IFN-β was detected only in rMP12-C13 type virus infected cells beginning at 6 h p.i., and peaking at 24 h p.i. In contrast, IFN-β secretion was not elevated in MP-12 or ZH501 infected BMDMs.

Next, we evaluated a panel of cytokines to identify potential points of response regulation by RVFV NSs (Fig. 3). In these studies we found that the overall expression levels of IFN-γ, IL-12(p70) and TNF-α were considerably lower in mock, MP-12 and ZH501 infected cells compared with rMP12-C13 type infected cells (Fig. 3a–c). At later time points, expression of these three cytokines and IL-1α (Fig. 3d) were generally higher in MP-12 infected cells when compared with mock or ZH501 infected cells, the latter of which failed to induce a significant cytokine response altogether. With all cytokines measured, the responses to rMP12-C13 type virus infection was significantly higher compared with MP-12 or ZH501 infected cells at virtually all time points (Fig. 3). These data suggest the RVFV NSs is critical for the ability of the virus to inhibit the cytokine response from infected mouse macrophages.

Furthermore, we examined a series of cytokines associated with the inflammatory or proliferative response to infection including IL-3, IL-4, IL-5, IL-6, IL-10 and IL-17 (Fig. 4). At all time points, infection with rMP12-C13 type virus resulted in a more pronounced response than did infection with MP-12 or ZH501 virus. However, at 72 h p.i. the response in MP-12 infected cells was either greater than (i.e. IL-10; Fig. 4b) or statistically equivalent (i.e. IL-3, IL-4 and IL-17; Fig. 4c, d, f) to the response seen in rMP12-C13 type infected cells. There was no significant increase in secretion of any of these six cytokines from ZH501 infected cells. These data further support the notion that the RVFV NSs is a significant regulator of the host immune response in BMDMs.

Chemokine response in primary mouse BMDMs

To evaluate chemotactic stimulation following RVFV infection of BMDM, we examined the secretion of chemokines during the course of infection. Similar to the cytokine response, infection with rMP12-C13 type virus induced a pronounced chemokine response that was significantly higher than other viruses at virtually all time points (Fig. 5). The chemokine response to MP-12 infection of BMDM was significantly higher than ZH501 infection with the release of RANTES, MCP-1, MIP-1α and KC (Fig. 5). These data demonstrate that RVFV strains expressing a functional NSs effectively interfere with the chemokine production of mouse macrophages. Similarly to some of the cytokines that were evaluated, it appears that BMDM can compensate for NSs induced inhibition of immune responses in MP-12 infected cells.
Intracellular signalling in RVFV infected primary mouse BMDM

To identify critical regulatory factors for cytokine or chemokine expression in RVFV infected BMDM, we performed a screen of major intracellular signalling cascades previously identified as regulators in cytokine and chemokine production. Here, we found that infection with rMP12-C13 type RVFV induced an increase in phosphorylated c-Jun (Ser63) (Fig. 6a) and p38 MAPK (Thr180/Tyr182) (Fig. 6b) at 1 and 6 h p.i. compared with MP-12 and ZH501. After MP-12 infection there were marked increases in phosphorylated STAT2 (Tyr689) (Fig. 6c), p53 (Ser15) (Fig. 6d), JNK (Thr183/Tyr185) (Fig. 6e) and NF-κB (p65) (Ser536) (Fig. 6f) at 48 and 72 h p.i. ZH501 infection induced only moderate and transient changes in the phosphorylation state of STAT2, p53 and JNK (Fig. 6c–e), but did induce significant phosphorylation of p38 MAPK at 24 h p.i. (Fig. 6b). These data support the role of NSs in regulating the host response by inhibiting components of intracellular signalling and further demonstrate an ability of ZH501 to block activation of intracellular signalling pathways.

Cytokine response in RAW 264.7 cells

As continuous cell lines provide a number of technical advantages over isolation and differentiation of primary cells, we were interested in determining the value of using an immortalized mouse macrophage cell line (RAW 264.7) to model infection of primary macrophages. Here, we found that the response of RAW 264.7 cells to infection with ZH501 or MP-12 resulted in an enhanced response relative to what was observed in primary cells.

**Fig. 3.** Cytokine release profile of mouse primary BMDM following RVFV infection. The concentration (pg ml$^{-1}$) of cytokines secreted by primary BMDM was measured after infection with MP-12 (grey striped bars), rMP12-C13 type (light grey bars) and ZH501 (black bars). Open bars represent mock-infected cells. Error bars represent ± SD from three individual biological replicates and *, # and + indicate P < 0.05 between mock/MP-12, mock/ZH501 or MP-12/ZH501, respectively, while **, ## and ++ indicate P < 0.05 between mock/rMP12-C13, MP-12/rMP12-C13 or ZH501/rMP12-C13, respectively. Cells were infected at an m.o.i. of 4.
Fig. 4. Interleukin release profile of mouse primary BMDM following RVFV infection. The concentration (pg ml⁻¹) of interleukins secreted by primary BMDM was measured after infection with MP-12 (grey striped bars), rMP12-C13 type (light grey bars) and ZH501 (black bars). Open bars represent mock-infected cells. Error bars represent ± SD from three individual biological replicates and *, # and + indicate P < 0.05 between mock/MP-12, mock/ZH501 or MP-12/ZH501, respectively, while **, ## and ++ indicate P < 0.05 between mock/rMP12-C13, MP-12/rMP12-C13 or ZH501/rMP12-C13, respectively. Cells were infected at an m.o.i. of 4.

IL-12(p70) (Fig. 7b) and TNF-α (Fig. 7c) were generally higher in mock, MP-12 and ZH501 infected RAW 264.7 cells when compared with BMDM (Fig. 3a–c). Infection with ZH501 induced a significant increase in IFN-γ levels at 36 h p.i. (Fig. 7a), while no apparent change could be detected in cells infected with MP-12.
IL-12(p70) expression appeared to be inhibited at early stages of infection (up to 6 h p.i.) with either MP-12 or ZH501 (Fig. 7b). At later time points, IL-12(p70) expression levels increased after ZH501 infection, while expression remained reduced in MP-12 infected cells (Fig. 7b). IFN-γ and IL-12(p70) are two key controlling cytokines for activation of Th1 immunity among other functions suggesting a differential modulation of Th1 activation by RAW 264.7 cells infected with either MP-12 or ZH501.

TNF-α expression was strongly induced in RAW 264.7 cells within 12 h p.i. in both MP-12 and ZH501 infected cells as compared with mock cells; however, the concentration of released TNF-α gradually declined throughout the course of the experiment (Fig. 7c). Interestingly, TNF-α levels were higher in MP-12 compared with ZH501 infected cells at all time points except 3 h p.i. The significantly elevated TNF-α levels could be correlated with the depression of IL-12(p70) since TNF-α has been shown to inhibit IL-12 production in macrophages (Ma et al., 2000).

**Chemokine response in RAW 264.7 cells**

The chemokine response in RVFV infected RAW 264.7 cells was characterized by analysing the expression profile of three chemokines which are frequently elevated during viral infections. For RANTES (Fig. 7d), MCP-1 (Fig. 7e) and MIP-1α (Fig. 7f), the concentration in mock cells increased over the first 12 h p.i. before gradually decreasing. In MP-12 infected cells, the secretion of those chemokines was significantly inhibited relative to both mock and ZH501 infected cells over the duration of the experiment. Chemokine levels released from ZH501 infected cells were reduced relative to mock at most time points, particularly in the case of MCP-1. However, infection with ZH501 did stimulate a more pronounced chemokine response than did infection with MP-12, suggesting
Fig. 6. Cell signalling profile of mouse primary BMDM following RVFV infection. Fold changes in the phosphorylation of key proteins after infection of primary BMDM after infection with MP-12 (grey striped bars), rMP12-C13 type (light grey bars) and ZH501 (black bars). Open bars represent mock-infected cells. Error bars are ±SD and *, # and + indicate $P < 0.05$ between mock/MP-12, mock/ZH501 or MP-12/ZH501, respectively, while **, ### and +++ indicate $P < 0.05$ between mock/rMP12-C13, MP-12/rMP12-C13 or ZH501/rMP12-C13, respectively. Cells were infected at an m.o.i. of 4.

differential regulation of the chemokine response by the two viruses. These data suggest a generalized inhibition of chemotactic responses in RVFV infected cells, particularly in MP-12 infected cells. In RAW 264.7 cells, the observed response contrasted greatly to what was observed in RVFV infected BMDM; therefore, immortalized RAW 264.7 cells appear not to be a good surrogate model for primary BMDMs.
DISCUSSION

The objective of this study was to evaluate the susceptibility and cellular responses of primary mouse macrophage cells to RVFV infection. In addition, we were interested in evaluating the suitability of an immortalized murine macrophage cell line for use as a surrogate for primary cells and whether the response to infection in cultured cells recapitulated primary cell responses. In our studies, we found that the response of RAW 264.7 cells to infection with ZH501 or MP-12 resulted in an enhanced response relative to responses observed in primary mouse cells.

Fig. 7. Cytokine and chemokine release profile in RAW 264.7 cells after RVFV infection. The concentration (pg ml^{-1}) of cytokines secreted by RAW 264.7 cells after infection with RVFV MP-12 (grey striped bars) or RVFV ZH501 (black bars). White bars represent mock-infected cells. Error bars represent ± so from three individual biological replicates and *, # and + indicate $P < 0.05$ between mock/MP-12, mock/ZH501 or MP-12/ZH501, respectively. Cells were infected at an m.o.i. of 1.
Both virus strains appeared capable of inhibiting components of the host response based on decreases in constitutive release of RANTES, MCP-1 and MIP-1x (Fig. 7d–f). There is also an apparent differential regulation between MP-12 and ZH501, where expression of RANTES, MCP-1 and MIP-1x is greater in ZH501 infected cells than MP-12 infected cells. These data suggest that ZH501 infection induces responses that could result in recruitment of T-cells and macrophages to the infection site. In the case of the pro-inflammatory cytokine TNF-α, MP-12 infection generally led to elevated concentrations relative to ZH501 infection, suggesting a more pronounced pro-inflammatory response in RAW 264.7 cells (Fig. 7c). Evaluation of Th1 related cytokines IFN-γ and IL-12(p70) indicated that ZH501 may elicit stronger Th1 responses relative to MP-12 infection in RAW 264.7 cells (Fig. 7a, b), but there are no obvious trends in expression making these data difficult to interpret. In total, there are differences in the response to RVFV MP-12 and ZH501 infection in RAW 264.7 cells, which would make them useful in efforts to characterize RVFV induced regulation of cytokine and chemokine release following infection; however, data generated in RAW 264.7 cells should be considered with caution as they may not accurately reflect the biological situation in primary macrophages.

The response to RVFV infection in primary mouse BMDM was quite different from what was observed in cultured RAW 264.7 cells. Inclusion of a recombinant MP-12 variant that is largely devoid of the viral NSs protein and resembles the clone 13 strain (rMP12-C13 type) (Ikegami et al., 2006) allowed for the identification of a specific role for NSs in regulating the host response in primary macrophages. Here, we did not observe the constitutive expression of chemokines as we did in RAW 264.7 cells. Instead, expression levels for each of the cytokines and chemokines evaluated, and at most time points, were higher in cells infected with MP12-C13 type than in ZH501 or MP-12 infected cells. In BMDM, most of the cytokines and chemokines were stimulated by rMP12-C13 type virus within 6 h p.i. (Figs 3–5), which is consistent with an inability of the virus to inhibit general activation of MAPK, p38 and c-Jun (Schoenborn & Wilson, 2007). As seen in Fig. 6(a, b), phosphorylation of c-Jun and p38-MAPK is upregulated at 1 and 6 h p.i. suggesting activation of the MAPK signalling pathways leading to IFN-γ release in rMP12-C13 type infected cells.

In order to identify potential mechanisms of viral regulation of the host response in RVFV infected primary BMDM, we evaluated a panel of phosphoproteins from intracellular kinase signalling cascades that are associated with regulation of host transcription and cell cycle control. Here we found evidence of differential regulatory mechanisms through the NSs protein. Infection with rMP12-C13 type induced a significant increase in levels of phosphorylated p38 MAPK and c-Jun, as indicated above, and a nominal increase in phosphorylated p-JNK at 1 and 6 h p.i. that was not replicated in cells infected with either MP-12 or ZH501 (Fig. 6e). Activation of both p38 MAPK and JNK can be induced by cellular stress or various cytokines, with many of the stimuli for activation being similar, and components of their signalling pathways being shared (Cargnello & Roux, 2011). The phosphorylation of both p38 and JNK with the subsequent phosphorylation of c-Jun early in infection suggests activation of MAPK kinase (MKK)4, which is able to phosphorylate both p38 and JNK (Cuadrado & Nebreda, 2010). Phosphorylation of Thr180/Tyr182 residues on p38 MAPK by MKK3, MKK4 or MKK6 generates the active form of p38, which is also capable of autophosphorylation (Askari et al., 2009; Kang et al., 2006; Remy et al., 2010). The ability of NSs containing RVFVs to prevent p38, JNK and c-jun activation early in the infection suggests a rapid inhibition of MKKs or other immediately upstream signalling proteins. The inhibition of p38 MAPK and c-Jun phosphorylation is complete with all three of the viruses tested while levels of activated JNK increase 6 h p.i. These data suggest that inhibition of MKKs, or related activation kinases, may be complete but that an alternative JNK activation pathway is enhanced in...
MP-12 infected BMDM, in particular. The early activation of c-Jun likely leads to increases in a number of cellular response genes, including IFN response genes (Gough et al., 2007). Phosphorylation profiles of JNK and p53 induced by RVFV infection are very similar following infection with each of the three individual viruses. Following ZH501 infection, there is a peak of phosphorylation at 24 h p.i. that suggests a transient activation of the JNK-p53 pathway. Activation of these proteins in MP-12 infected cells begins at 48 h p.i. and is sustained through 72 h p.i., slightly delayed relative to observations made by Austin et al. (2012) for phosphorylation of p53 (Ser\textsuperscript{15}) in RVFV infected airway epithelial cells where both JNK and p53 were transiently phosphorylated at 24 and 48 h p.i. Our finding that p53 was phosphorylated following infection with the rMP12-C13 type virus, which lacks NSs, is in conflict with Austin et al. (2012), who suggest that p53 phosphorylation is NSs dependent; however, different cell types were used in the two studies suggesting a cell type dependent response. JNK is an upstream regulator of p53 in a pathway that can be activated by either growth factors or cytokines suggesting that the delayed activation of this pathway may be due to stimulation by pro-inflammatory cytokines, several of which are induced in MP-12 infected BMDM at 48–72 h p.i. (Fig. 3). The fact that c-Jun does not show a concomitant increase in the phosphorylation state suggests that the downstream genes regulated via the JNK-p53 pathway at 48–72 h p.i. are of a distinct subset that leads to cell cycle arrest or apoptosis. Our studies evaluated increases in Ser\textsuperscript{15} phosphorylation in the transactivation domain of p53 which is a target of several kinases and which facilitates binding of the p300/CBP (cAMP response element binding protein) complex to stimulate the transactivation function of p53 (Meek, 2009). Activation of p53 has also been shown to induce gene expression of both Toll-like receptor (TLR)3 and TLR9, which contain pattern recognition receptors for dsRNA and viral RNA, respectively (Kawai & Akira, 2007; Menendez et al., 2011). Enhancing TLR3 or TLR9 expression would enhance the antiviral response within BMDM. These data suggest that NSs containing viruses may inhibit activation of the JNK-p53 pathway, but that any inhibition is either transient or superseded by other pathways.

To evaluate STAT signalling following RVFV infection, we measured phosphorylation levels of STAT2 (Tyr\textsuperscript{699}) (Fig. 6c) and STAT3 (Tyr\textsuperscript{705}) (data not shown). Phosphorylation levels of STAT3 were essentially unchanged, while levels of STAT2 significantly increased at 72 h p.i. The phosphorylation of STAT2 and not STAT3 is indicative of activation of specific intracellular pathways. STAT2 is a critical component of type I IFN signalling, but is not thought to participate in TLR associated signalling (Alazawi et al., 2013). STAT2 signalling is induced predominantly by type I and type III IFN receptors suggesting that activation of STAT2 in our studies is due to autocrine IFN signalling. A number of viruses have shown the ability to antagonize STAT2 signalling through various means including indirect inhibition, degradation of protein or mRNA and blocking STAT2 tyrosine phosphorylation (Chowdhury & Farrar, 2013). STAT2 accumulation in BMDM is indicative of a type I IFN response as STAT2 phosphorylation is activated by binding of IFN-α/β or IFN-λ to IFNAR and IFNLR, respectively, followed by JAK1/TYK2 phosphorylation of STAT2.

The data presented here support the hypothesis that attenuating mutations within RVFV MP-12 impact the ability of the virus to inhibit host antiviral responses. The WT RVFV ZH501 is able to maintain a more sustained inhibition of host cytokine secretion and kinase activation cascades than the MP-12 vaccine virus. Evaluation of rMP12-C13 type virus, which is devoid of functional RVFV NSs protein, found that this virus lacks inhibitory mechanisms present in MP-12 and ZH501 viruses, but that some suppression of the host response in BMDM remains intact. Our data indicate that NSs is a significant controller of host cytokine and chemokine release beyond the regulation of type I IFN, which has previously been established (Billecocq et al., 2004; Bouloy et al., 2001; Ermler et al., 2014). The viral NSs protein has been shown to stimulate degradation of PKR leading to inhibition of p53 and eIF2α activation, the latter activity effectively suppresses eIF2α regulated general host transcription (Ikegami et al., 2009a; Kalveram et al., 2013). Although both MP-12 and ZH501 viruses have identical NSs proteins, the ability of ZH501 to more completely suppress the systemic host response suggests that other viral proteins, such as NSm, could also play a role in regulating the response to infection.

**METHODS**

**Cells.** Immortalized mouse macrophage cells (RAW 264.7, ATCC# TIB-71) and VERO C1008 (Vero E6) cells (ATCC # CRL-1586) were maintained in Dulbecco’s modified Eagle’s media (DMEM) and supplemented as recommended by ATCC and kept at 37 °C and 5 % CO\textsubscript{2}.

BMDMs were isolated from adult (8–10 week old) female C57BL/6 mice. After euthanasia, femurs were removed and stripped of muscle, exposed bone was washed in 70 % EtOH and rinsed with RPMI media with 1 % penicillin/streptomycin and 10 % FBS (cRPMI) and then manually separated with two 23-gauge needles. Cells were pelleted by centrifugation at 300 g for 7 min and 4 °C, and the cell pellet was resuspended in 5 ml red blood cell lysis buffer (eBioscience). Next, RPMI-PS was added to the cell solution and centrifugation repeated. The cell pellet was washed twice with RPMI, resuspended in cRPMI and plated at a density of 2 x 10\textsuperscript{5} cells dish\textsuperscript{-1} in 60 ml of cRPMI media supplemented with 20 ng ml\textsuperscript{-1} of M-CSF (eBioscience). After 3 days, half the volume of media was replaced. After four additional incubation days, cells were washed with PBS and 3 ml of non-enzymic cell dissociation buffer (Sigma, catalogue #C5914) was added. Following 10 min at 37 °C and 5 % CO\textsubscript{2}, cells were removed, centrifuged at 300 g for 7 min at 4 °C and then plated for infection or lipopolysaccharide (LPS) stimulation.

**Viruses.** Pathogenic RVFV WT strain ZH501 and the original live-attenuated vaccine strain MP-12 were obtained from Drs C.J. Peters...
and John Morrill (University of Texas Medical Branch, UTMB). Recombinant attenuated RVFV rMP12-C13 type was kindly provided by Dr Tetsuro Ikekami (UTMB) (Ikekami et al, 2006) and resembles the Clone13 strain by containing the 70 % in-frame deletion mutation of the NSs gene. This virus was created on the backbone of the original live-attenuated MP-12 vaccine strain. All viruses were cultured and titrated on Vero E6 cells. For infection of cells, virus was diluted in DMEM supplemented with 4 % FBS. All work involving infectious ZH501 was performed in the Robert E. Shope or Galveston National Laboratory BSL-4 laboratories (UTMB). Cell supernatant samples removed from BSL-4 containment for analysis at BSL-2 were inactivated by γ-irradiation (5 Mrad). Phosphoprotein analysis of cell lysates was performed within BSL-4 on a Bio-Plex 200 system (Bio-Rad).

**Virus titration.** Vero E6 cells were infected with serial 10-fold dilutions of virus containing samples for 1 h and then overlain with a tragacanth (0.8 %)/MEM overlay with 2 % FBS and 1 % penicillin/streptomycin. After 4 days, the overlay was removed and cells were stained with 0.2 % crystal violet diluted in 10 % neutral buffered formalin. Plates were enumerated and viral titres reported as log_{10} p.f.u. For rMP12-C13 type, infected Vero E6 cells were overlain with 0.6 % noble agar (VWR) supplemented with 10 % FBS, 1 % penicillin/streptomycin and 10 % treptose phosphate broth (MP Biomedicals). After 3 days, a second overlay containing 0.35 % neutral red was directly added to the initial overlay. The plates were incubated for an additional 24 h, plates enumerated and the viral titres were reported as log_{10} p.f.u. (Kalveram et al, 2011).

**Macrophage infection.** Primary macrophages and macrophage cell lines were infected at an m.o.i. of 4 or 1, respectively, in biological triplicates using the same virus inoculum. After infection, cells were incubated at 37 °C/5 % CO₂ for 1 h with rocking of the plates every 15 min. Cells were then washed three times with PBS and covered with cRPMI for primary cells and fresh DMEM media supplemented with 4 % FBS and 1 % non-essential amino acids (cDMEM) for RAW 264.7 cells. Cell culture supernatants were harvested at pre-defined times after infection and stored at −80 °C until analysis.

**Bio-Plex assay.** Irradiated cell culture supernatants were processed following the manufacturer’s instructions (Bio-Rad) and analysed on a Bio-Plex 200 system (Bio-Rad). Pre-designed mouse 23-plex cytokine assays and 8-plex phosphoprotein assays were utilized. The cytokine assays measured the concentration of cytokines, while the phosphoprotein assays reported data in fold change over mock cells. In primary cells, LPS (50 μg ml⁻¹) was used as a positive control, and cell supernatants analysed at 48 h after LPS stimulation to demonstrate cell responsiveness (data not shown) (Chia et al, 1989; Pan et al, 2008; Yi et al, 2013).

**IFN-β ELISA.** Irradiated cell culture supernatants were analysed using the sandwich ELISA method following the manufacturer’s instructions (PBL Interferon Source). The plate was read on a micro-titre plate reader at a wavelength of 450 nm (Bio-Rad).

**Statistical analysis.** A paired Student’s t-test (P>0.05 for a 95 % confidence interval) and a one-way ANOVA were used to analyse data. A t-test correction (P>0.05 for a 95 % confidence interval) was used to determine statistical significance between two different groups.

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