Critical role for bone marrow stromal antigen 2 in acute Chikungunya virus infection

Wadie D. Mahauad-Fernandez, Philip H. Jones and Chioma M. Okeoma

1Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA
2Interdisciplinary Graduate Program in Molecular and Cellular Biology (MCB), University of Iowa, Iowa City, IA, USA

Bone marrow stromal antigen 2 (BST-2; also known as tetherin or CD317) is an IFN-inducible gene that functions to block the release of a range of nascent enveloped virions from infected host cells. However, the role of BST-2 in viral pathogenesis remains poorly understood. BST-2 plays a multifaceted role in innate immunity, as it hinders retroviral infection and possibly promotes infection with some rhabdo- and orthomyxoviruses. This paradoxical role has probably hindered exploration of BST-2 antiviral function in vivo. We reported previously that BST-2 tethers Chikungunya virus (CHIKV)-like particles on the cell plasma membrane. To explore the role of BST-2 in CHIKV replication and host protection, we utilized CHIKV strain 181/25 to examine early events during CHIKV infection in a BST-2−/− mouse model. We observed an interesting dichotomy between WT and BST-2−/− mice. BST-2 deficiency increased inoculation site viral load, culminating in higher systemic viraemia and increased lymphoid tissues tropism. A suppressed inflammatory innate response demonstrated by impaired expression of IFN-α, IFN-γ, and CD40 ligand was observed in BST-2−/− mice compared with the WT controls. These findings suggested that, in part, BST-2 protects lymphoid tissues from CHIKV infection and regulates CHIKV-induced inflammatory response by the host.

INTRODUCTION

Infection with the alphavirus Chikungunya virus (CHIKV) causes disease in a wide range of animals and humans following infected mosquitoes bites (Pialoux et al., 2007). Clinical manifestations of infection include fever, skin rash, myalgia, and polyarthralgia accompanied by swollen ankles, knees and wrists (Powers & Logue, 2007). Although the mechanisms for viral pathogenesis and host protection are poorly understood, the host immune/inflammatory responses are thought to mediate viral clearance and, in most cases, initiate subsequent CHIKV-induced arthralgia/arthritis sequelae. Many human and murine cells are susceptible to CHIKV infection, and may play a role in CHIKV-induced pathology (Labadie et al., 2010). Among these are myeloid and lymphoid cells, fibroblasts, and epithelial cells (Das et al., 2010; Her et al., 2010; Labadie et al., 2010; Sourisseau et al., 2007; Wikan et al., 2012). The susceptibility of various host cells/tissues to CHIKV is beginning to unravel; however, the factors that regulate CHIKV tropism are poorly understood.

Host innate cellular factors have been shown to predict response to CHIKV (Olagnier et al., 2014; Partidos et al., 2011; Schilte et al., 2010; Seymour et al., 2013; Wauquier et al., 2011; Werneke et al., 2011). Signalling through type 1 IFN receptor (IFN-α/βR) plays a role in controlling CHIKV infection and CHIKV-induced arthritogenic disease (Briolant et al., 2004; Couderc et al., 2008, 2009; Gardner et al., 2012; Partidos et al., 2011; Schilte et al., 2010). Nonetheless, IFN-α/βR-independent control of CHIKV has been reported (Olagnier et al., 2014; Partidos et al., 2011). Our previous studies revealed that the host restriction factor (HRF) called bone marrow stromal antigen 2 (BST-2; also known as tetherin or CD317) retains budding CHIKV virus-like particles on the cell membrane and that CHIKV non-structural protein 1 (nsP1) counteracted BST-2-mediated tethering of CHIKV (Jones et al., 2013b), signifying that BST-2 may be an essential HRF against CHIKV.

BST-2 is an IFN-inducible factor that tethers various nascent enveloped viruses on the cell surface (Douglas et al., 2010; Jones et al., 2012, 2013b; Lopez et al., 2012; Neil et al., 2008; Radoshitzky et al., 2010). In addition to tethering, BST-2 plays a multifaceted role in innate immunity by possibly promoting infection with vesicular stomatitis virus, a rhabdovirus (Swiecki et al., 2012), orthomyxoviruses such as influenza B virus (Swiecki et al., 2012), and inhibiting replication of retroviruses as in human
immunodeficiency virus type 1 (HIV-1) (Neil et al., 2008), mouse mammary tumor virus (Jones et al., 2012) and murine leukemia virus (Liberatore & Bieniasz, 2011). BST-2 may mediate its antiviral effect by modulating the induction of pro-inflammatory gene expression through NFκB (Galão et al., 2012; Tokarev et al., 2013) and inducing antibody-dependent cell cytotoxicity (Arias et al., 2014; Pham et al., 2014). These multifaceted and probably contradictory roles have hindered exploration of BST-2 antiviral function in vivo.

In the present study, we undertook experiments to probe the role of BST-2 in acute CHIKV replication and host protection. We utilized the live-attenuated CHIKV 181/25 strain developed at the US Army Medical Research Institute of Infectious Diseases (Levitt et al., 1986) that produces non-lethal infection in WT mice (Gardner et al., 2012) and can be safely used under biosafety level 2 conditions. This model allows evaluation of CHIKV-mediated alteration of host response in immunocompromised mice. As early events are more likely to be causal than events that are observed after the onset of disease symptoms and sequelae, our studies were conducted 24 h post-infection. We found that BST-2 deficiency was associated with high systemic viraemia, increased virus spread to distant tissues, enhanced lymphoid tissues tropism, and suppressed expression of IFN-α, IFN-γ and CD40 ligand (CD40L).

**RESULTS**

**BST-2 restricts release of CHIKV 181/25 from both human and murine cells**

We reported previously that BST-2 retains CHIKV virus-like particles on the cell surface (Jones et al., 2013b). To determine whether BST-2 had any effect on the release of infectious virions, we performed virus release assays in different human cells infected with CHIKV 181/25 (m.o.i. 0.1). HeLa cells expressing high BST-2 produced significantly less virus compared with 293T cells expressing less BST-2 as determined by end-point dilution assay (EPDA) (Figs 1a–c and S1, available in the online Supplementary Material). Treatment of HeLa cells with IFN-α had no effect on BST-2 expression (Fig. 1a), but IFN-α increased BST-2 expression in 293T cells (Fig. 1b). The absence of an IFN-α effect on BST-2 induction in HeLa cells correlated with no change in virus release, whilst the increase in BST-2 expression in 293T cells correlated with a decrease in the amount of virions released into the extracellular milieu (Fig. 1c). These results suggested that induction of BST-2 with IFN-α had the potential to block CHIKV release from epithelial-like 293T cells.

To evaluate if IFN-α-induced BST-2 would have any effect on myeloid-like cells, human monocytic U937 cells were treated with PBS or IFN-α followed by infection with CHIKV. U937 cells, like other myeloid and lymphoid cells,

express BST-2 (Fig. S1) and treatment with IFN-α enhanced BST-2 expression (Fig. 1d). Upon infection with CHIKV, U937 cells treated with IFN-α released fewer virions into the culture medium compared with cells treated with PBS (Fig. 1e). These data suggested that endogenous BST-2 induced by IFN-α may play a role in inhibiting CHIKV release from infected U937 cells. It appeared that the steady level of BST-2 in HeLa, 293T and U937 cells may have contributed to their ability to respond to CHIKV infection.

Next, we pre-treated immortalized WT and BST-2−/− mouse embryonic fibroblasts (MEFs) with PBS or IFN-β prior to infection, and showed that IFN-β induced BST-2 expression in immortalized WT MEFs, but not in BST-2−/− MEFs (Fig. 1f). The induction of BST-2 in WT MEFs resulted in inhibition of virus release (Fig. 1g) as determined by EPDA. As EPDA only measured infectious virus, we sought to determine the total viral titre (number of infectious and non-infectious viruses) produced by each genotype. For this purpose, we employed the nanoparticle tracking analysis (NTA) method. Using the NTA system (Du et al., 2010; Filipe et al., 2011), we directly visualized, sized and counted total CHIKV particles in culture medium from WT and BST-2−/− MEFs pre-treated with PBS or IFN-β. As expected, the virus titre from NTA was higher than the EPDA titre (compare Fig. 1g, h). However, similar to the EPDA results, expression of BST-2 prevented CHIKV release from MEFs, and IFN-β treatment of WT MEFs further enhanced BST-2-mediated inhibition of CHIKV release with no significant effect on BST-2−/− MEFs (Fig. 1h).

To validate our findings, we treated primary WT and BST-2−/− MEFs with PBS or IFN-β (Fig. 1i) followed by infection. Using NTA, we showed that culture supernatants from BST-2−/− MEFs contained a significantly higher virus titre regardless of IFN-β treatment, whilst supernatants from WT MEFs had a lower virus titre, which was further reduced in the presence of IFN-β (Fig. 1j). Furthermore, expression of BST-2 in primary macrophages was evaluated with confocal microscopy. We showed that CHIKV proteins colocalized with BST-2, as highlighted in the enlarged merged image (Fig. 1k). In macrophages, BST-2 inhibited CHIKV release from infected WT cells (Fig. 1l), although with reduced efficiency compared with MEFs (compare Fig. 1j, l). It should be noted that a modest non-significant decrease in virus release was observed in IFN-treated BST-2−/− cells compared with vehicle-treated BST-2−/− cells (Fig. 1g, h, j, l). This difference could be attributed to a BST-2-independent antiviral effect of IFNs against CHIKV, as has been reported previously (Briolant et al., 2004; Couderc et al., 2008, 2009; Gardner et al., 2012; Partidos et al., 2011; Schilte et al., 2010). These results suggested that BST-2 expression significantly impaired CHIKV 181/25 release.

**BST-2-deficient mice are highly susceptible to CHIKV replication at the site of inoculation**

The effect of BST-2 on CHIKV replication was tested in a subcutaneous footpad infection assay. Inoculation of WT mice
Fig. 1. BST-2 blocks CHIKV release in tissue culture cells. (a, b) Surface BST-2 expression in HeLa (a) and 293T (b) cells cultured in the presence and absence of IFN-α by FACS analysis. (c) EPDA of CHIKV titre released by HeLa and 293T cells cultured in the presence and absence of IFN-α. (d) Surface BST-2 expression in U937 cells treated with or without IFN-α. (e) EPDA of CHIKV titre released by U937 cells treated with or without IFN-α. (f) IFN-β-mediated induction of BST-2 mRNA in immortalized WT MEFs as determined by quantitative real-time (qRT)-PCR. (g) CHIKV titre released by WT and BST-2−/− MEFs cultured in the presence and absence of IFN-β as determined by EPDA. (h) NTA of CHIKV titre released by WT and BST-2−/− MEFs cultured in the presence and absence of IFN-β. (i) Expression of BST-2 mRNA in primary WT MEFs treated with or without IFN-β as determined by NTA. (k) Confocal imaging of BST-2 (green) and CHIKV (red) expression in primary WT macrophages showing co-localization of CHIKV with BST-2. DAPI stains the nucleus and is in blue. Bar, 10 μm. (l) CHIKV titre released by primary WT and BST-2−/− macrophages treated with or without IFN-α as determined by EPDA. There was a significant increase in CHIKV release by BST-2−/− cells compared with WT cells. Treatment with IFNs reduced the amount of released CHIKV. Note that there was a slight decrease in CHIKV released by IFN-treated BST-2−/− cells, which could be attributed to a BST-2-independent IFN effect. For FACS analysis of BST-2 induction by IFN, each cell line was compared with the untreated cells. Virus titre of infectious fluid was determined by either EPDA (p.f.u. ml−1) or NTA (particles ml−1). Experiments were repeated several times with similar results and data presented as the mean±sd. Significance: *P<0.05 and **P<0.01; NS, not significant.
and BST-2−/− mice with CHIKV resulted in a significant increase in footpad tissue viral load. Analysis of CHIKV negative-strand RNA in five independent experiments showed that CHIKV replicated more efficiently (~10-fold) in the absence of BST-2 expression (Fig. 2a). Indeed, loss of one BST-2 allele was enough to render mice more susceptible to infection compared with mice with two BST-2 alleles (Fig. 2b). We did not find hind limb swelling in WT or BST-2−/− mice despite the robust virus replication in the footpad of BST-2−/− mice. This finding was not surprising as it had been shown previously that infection with CHIKV 181/25 did not induce hind limb swelling (Gardner et al., 2012). Our results indicated that expression of BST-2 inhibits local CHIKV replication.

**Deficiency of BST-2 increases systemic viraemia**

Given that the virus titre in the inoculation site in BST-2−/− mice was 10-fold higher than in WT, we hypothesized that the high viral load in BST-2−/− mice would enhance plasma viraemia. EPDA revealed that Vero cells infected with plasma from BST-2−/− mice succumbed to CHIKV-induced cytopathicity at a significantly higher rate than cells infected with BST-2+/− and WT plasma, in that order (Fig. 2c), suggesting that in an equal volume of plasma, BST-2−/− mice produced more infectious particles than BST-2+/− and WT mice (P<0.01). To confirm the viral titre result from EPDA, we quantified CHIKV RNA in WT and BST-2−/− cell-free plasma. We detected more CHIKV positive-strand RNA in BST-2−/− plasma compared with WT plasma (Fig. 2d). Our data indicated that BST-2 deficiency enhanced accumulation of viral particles in the bloodstream, suggesting that the higher viraemia in BST-2−/− mice may result in more systemic virus dissemination.

**BST-2−/− mice have a higher viral load in various peripheral tissues, except the heart**

To determine whether higher systemic viraemia in BST-2−/− mice would result in efficient infection of peripheral tissues, we examined the viral load in various lymphoid and non-lymphoid tissues. We found elevated levels of CHIKV RNA in the peripheral blood mononuclear cells (PBMCs), lymph nodes and spleen in BST-2−/− mice compared with their WT counterparts (Fig. 3a–c). Interestingly, the lymph nodes of BST-2−/− mice (Fig. 3b) were overly susceptible to CHIKV, more than the PBMCs (Fig. 3a) and spleen (Fig. 3c). Similar to lymphoid tissues, BST-2−/− mice had a higher viral RNA in the liver (Fig. 3d), lung (Fig. 3e) and stomach (Fig. 3f), but not in the heart (Fig. 3g), compared with WT mice. These data indicated that BST-2 had a tissue-specific effect on CHIKV replication.

**BST-2 expression modulates lymphoid tissue tropism in CHIKV infection**

Following skin infection, CHIKV spreads to other target tissues/organs within hours, infecting haematopoietic cells and non-haematopoietic cells, albeit to a lower extent (Ozden et al., 2007; Sourisseau et al., 2007). To evaluate the kinetics of the BST-2-dependent response to infection, we infected WT and BST-2−/− mice, and analysed the level of CHIKV RNA in various tissues (Fig. 4a) compared with the level in their respective inoculation site, i.e. footpad tissue (Fig. 4a, inset). The ability of CHIKV to infect different tissues depended on the host genotype. Generally, when compared with the viral load in footpad tissue, the tropism rate of CHIKV to the liver, lung and stomach was similar in both WT and BST-2−/− mice. In the heart, BST-2 deficiency protected mice from CHIKV infection. WT mice had twofold more viral RNA in the heart compared with BST-2−/− mice. Remarkably, the lymph nodes, PBMCs and spleen of BST-2−/− mice were highly permissive to CHIKV replication. To confirm these findings, we quantified the amount of infectious virus in the lymph nodes and spleen by EPDA using tissue homogenates. As expected, loss of BST-2 correlated with increased sensitivity of the lymph nodes and spleen (Fig. 4b, c) to infection. These results suggested that intrinsic BST-2 expression determined permissibility of lymphoid tissues to CHIKV infection.
BST-2 expression at the site of virus inoculation is regulated by CHIKV

To determine if BST-2 expression was altered by CHIKV infection, we evaluated BST-2 mRNA in the footpad tissue of naive and infected WT mice. BST-2 mRNA was elevated in WT footpad tissue 24 h post-infection (Fig. 4d). As BST-2 expression is induced by IFN-α, we examined IFN-α mRNA expression in the footpad tissue. BST-2−/− mice have higher basal IFN-α in their footpad tissue than WT mice (Fig. 4e). However, upon infection, CHIKV induced IFN-α mRNA expression in WT footpad tissue, but not in BST-2−/− footpad tissue (Fig. 4f), supporting a previous report that BST2−/− mice produced less type I IFN than their WT control following viral infection (Swiecki et al., 2012). The increase in footpad tissue IFN-α correlated with upregulation of BST-2 mRNA, indicating that CHIKV regulation of IFN-α was partly dependent on BST-2 expression.

Analysis of differentially expressed inflammatory cytokines and chemokines

Infection with CHIKV elicits a robust inflammatory response (Hoarau et al., 2010; Wauquier et al., 2011) resulting in alteration in the host transcriptome. Such alterations are likely determinants of the fate of infection, with regard to immune responses and disease outcome. To determine if the absence of BST-2 expression modulated inflammatory cytokines at the site of inoculation, we conducted targeted mouse inflammatory cytokine and receptor PCR array analysis using footpad tissue from naïve and infected mice. Out of 86 genes evaluated (Fig. 5a), 60 genes were commonly upregulated more than twofold in WT and BST-2−/− mice, and 17 genes were commonly downregulated (Fig. 5b). Nicotinamide phosphoribosyltransferase (NAMPT, also known as visfatin) was significantly downregulated in WT mice compared with BST-2−/− mice (Fig. 5b). We found that IFN-γ and CD40L were the most highly CHIKV-induced genes in WT mice compared with BST-2−/− mice. We validated IFN-γ and CD40L expression by quantitative real-time (qRT)-PCR. Basal IFN-γ mRNA was higher in BST-2−/− footpad tissue compared with WT footpad tissue (Fig. 5d), but CHIKV suppressed IFN-γ expression in BST-2−/− footpad tissue (Fig. 5e). The level of CD40L in naïve footpad tissue was similar between WT and BST-2−/− footpad tissue (Fig. 5f). However, CD40L mRNA significantly decreased in infected BST-2−/− footpad tissue (Fig. 5g). These results indicated that differences in early immune responses between WT and BST-2−/− mice may have been linked to their different responses to infection with CHIKV.

Expression of innate immunity genes in the lymph node draining the site of CHIKV delivery is BST-2 dependent

Here, we analysed the expression of IFN-γ, CD40L, IFN-α and BST-2 in the draining lymph nodes of WT and BST-2−/− mice. The basal level of IFN-γ present in BST-2−/− lymph nodes was significantly higher than that in the WT
controls (Fig. 6a). However, CHIKV suppressed IFN-γ expression in BST-2−/− lymph nodes (Fig. 6b) as well as IFN-γ production in BST-2−/− plasma (Fig. 6c). Likewise, basal CD40L expression (Fig. 6d, e) was higher in BST-2−/− lymph nodes, but CHIKV markedly reduced CD40L in BST-2−/− mice compared with the WT (Fig. 6f–g).

BST-2 expression in draining lymph nodes of WT mice was downregulated by CHIKV at both the mRNA (Fig. 6h) and protein (Fig. 6i) levels. Additionally, IFN-α mRNA was higher in naive BST-2−/− lymph nodes compared with WT lymph nodes (Fig. 6j). However, CHIKV significantly suppressed IFN-α expression in BST-2−/− lymph nodes, but not in WT (Fig. 6k). These results clearly indicated that BST-2 was critical for the protection of lymphoid tissues against CHIKV infection by promoting some early innate immune response events following infection.
DISCUSSION

In this study, we conducted in vivo experiments to improve our understanding of the role of BST-2 in CHIKV replication and host defence. We extended our prior work on the effect of BST-2 expression in the release of CHIKV virus-like particles (Jones et al., 2013b) and demonstrated that infectious CHIKV 181/25 is susceptible to the effect of BST-2 in human cells (Fig. 1c, e). We confirmed that infectious CHIKV co-localizes with BST-2, and that BST-2 inhibits release of infectious CHIKV from infected MEFs and macrophages (Fig. 1g, h, j, l).

It has been shown previously that CHIKV 181/25, which does not cause serious inflammatory disease, is non-lethal in mice (Gardner et al., 2012). In the present study, we did not observe lethality as we concluded all studies at 24 h. Despite the lack of lethality, BST-2−/− mice were highly susceptible to CHIKV replication at the site of inoculation. Indeed, viral load in the footpad tissue of infected BST-2−/− mice was 10-fold higher than that in WT controls (Fig. 2a). This point was further elucidated by the fact that heterozygote mice (BST-2+/-) were more susceptible to inoculation site CHIKV replication compared with WT mice (Fig. 2e). Increased inoculation site viral load in BST-2−/− mice correlated directly with amplified systemic viraemia 24 h post-infection, indicating that BST-2 mediates release of virions into the extracellular milieu in vivo.

The high extracellular virus titre in BST-2−/− mice corresponded to systemic dissemination and elevated viral...
BST-2 expression was found to be particularly important in protecting lymphoid tissues from CHIKV tropism. This could, in part, explain why lymphoid tissues in WT mice are relatively less susceptible to CHIKV infection, as has been reported previously (Das et al., 2010; Her et al., 2010; Labadie et al., 2010; Sourisseau et al., 2007). Interestingly, not all tissues lacking BST-2 are susceptible to high CHIKV infection. We found that loss of BST-2 protects the heart from CHIKV replication because levels of CHIKV negative-strand RNA present in the heart of WT mice were higher compared with their BST-2^{−/−} littermates, suggesting that BST-2 plays a tissue/cell-type-dependent role against CHIKV infection.

Evaluation of gene expression following CHIKV infection revealed that CHIKV altered the levels of inflammatory cytokines and chemokines in a BST-2-dependent manner. We found that levels of NAMPT were downregulated in WT mice infected with CHIKV and slightly upregulated (1.9-fold) in CHIKV-infected BST-2^{−/−} mice. The finding in WT mice supports a previous report which showed that
CHIKV infection downregulated NAMPT expression (Thio et al., 2013). NAMPT is located both intracellularly and extracellularly. Inside the cell, NAMPT functions as a rate-limiting enzyme in the NAD⁺ salvage pathway (Garten et al., 2009). Extracellular NAMPT is suggested to have enzymic and pro-inflammatory activities (Romacho et al., 2009; Wang et al., 2009). NAMPT has been implicated in various pathological conditions, such as rheumatoid arthritis (Otero et al., 2006). In HIV-1-infected cells, suppression of NAMPT expression by miR-182 was shown to be involved in Tat-induced HIV-1 long-terminal repeat transactivation (Chen et al., 2013). In addition, NAMPT inhibited infection by HIV-1 using the CCR5 co-receptor, but enhanced infection by CXCR4 (Van den Bergh et al., 2012). The effect of NAMPT on HIV-1 replication suggests that NAMPT has the potential to selectively modulate viral pathogenesis. The differential effect of CHIKV on NAMPT levels in the presence and absence of BST-2 is interesting and indicative of BST-2-mediated regulation of NAMPT.

Another significant finding from our study was the suppression of IFN-α and IFN-γ by CHIKV in BST-2−/− mice. Previous studies indicated that BST-2 blocks secretion of IFN-α by plasmacytoid dendritic cells (Cao et al., 2009), and we found mRNAs encoding IFN-α and IFN-γ to be higher in BST2−/− mouse footpad and lymph node tissues at baseline compared with WT mice. The reason behind the increase in steady-state levels of IFN-α and IFN-γ in uninfected BST-2−/− mice is unclear, but could be attributed to compensatory mechanisms for immune regulation in the absence of BST-2. Upon infection, however, CHIKV downmodulates the expression of these IFNs, supporting a previous finding that BST2−/− mice produced less IFN-α than their WT controls following systemic viral infections (Briolant et al., 2012). As type I and II IFNs are critical for the control of CHIKV infection (Briolant et al., 2004; Couderc et al., 2008, 2009; Gardner et al., 2012; Partidos et al., 2011; Schilte et al., 2010; Wauquier et al., 2011), our finding implies that the inability of BST-2−/− mice to produce IFNs upon infection with CHIKV may contribute to their enhanced susceptibility to the virus. Furthermore, the absence of IFN mRNA and protein in BST-2−/− mice is suggestive of the involvement of BST-2 in the regulation of IFN during CHIKV infection. This point is intriguing as BST-2 has the potential to activate antigen-presenting cells (APCs), as described previously (Blasius et al., 2006). In this case, BST-2 expressed in WT cells may function to sense CHIKV and prime target cells in response to CHIKV, with a resultant increase in cytokine secretion.

In addition to suppression of IFNs in BST-2−/− mice, we found that expression of CD40L in BST-2−/− mice was suppressed markedly by CHIKV. CD40L is a type II transmembrane inflammatory regulator expressed in many cell types, including CD4⁺ T-cells, monocytes, macrophages, endothelial cells and platelets (Rizvi et al., 2008). As with IFNs, an enhanced basal CD40L level in BST-2−/− mice could be a compensatory event for the generation of optimal immune activation. However, CHIKV was able to downmodulate CD40L in BST-2−/− mice. The reduction of CD40L in infected BST-2−/− mice could stem from the inability of BST-2−/− mice to efficiently sense incoming CHIKV, resulting in inefficient APC activation, suboptimal CD40–CD40L interaction and secretion of cytokines, including IFN-γ. Indeed, BST-2 has been shown recently to act as a pattern recognition receptor that senses HIV-1 and activates the NFκB signalling pathway, culminating in secretion of antiviral factors (Galão et al., 2012). A previous study implicated reduced CD40L and IFN-α expression in the pathogenesis of chronic CHIKV-induced arthritis (Malvy et al., 2009). CD40L can regulate the immune response (Mackey et al., 1998) either by (i) activating APCs to express co-stimulatory molecules including B7 (CD80 and CD86), (ii) generating cytotoxic T-lymphocytes against virus-infected cells or (iii) stimulating IFN-γ production.

The observed suppression of expression of IFN-α, IFN-γ and CD40L at the site of virus inoculation and in the popliteal lymph node draining the site of infection in BST-2−/− mice signifies that BST-2 regulates these genes. Deficiency of BST-2 expression results in suppression of genes that have been shown to be effective against CHIKV. Particularly affected may be genes that are involved in B-cell differentiation, optimal immunoglobulin isotype switching and antiviral activity. It is therefore possible that (i) CHIKV may more efficiently evade T-cell-dependent immunoglobulin (IgA and IgG) responses in the absence of CD40L gene expression (Whitmire et al., 1999), (ii) the presence of CD40L in WT mice may function to stimulate production of IFN-γ, which in turn protects cells from CHIKV infection, as has been reported for HIV (Fan et al., 1994; Kornbluth et al., 1989), and (iii) tethering of CHIKV at the cell surface by BST-2 (Jones et al., 2013b) may not only prevent CHIKV release, but also mediate clearance of the virus and its reservoirs through antibody-dependent cell-mediated cytotoxicity, as has been shown for HIV-infected cells (Arias et al., 2014; Veillette et al., 2014). Overall, our study has contributed to defining the effect of BST-2 on CHIKV replication and host protection. Further studies will be necessary to determine whether BST-2 is operative on more virulent CHIKV strains, assess the role of BST-2 in CHIKV disease and elaborate on the role of CHIKV-modulated genes in CHIKV pathogenesis in the absence of BST-2.

METHODS

Animals. WT and BST-2−/− mice (Jones et al., 2013a; Szczecki et al., 2012) were inoculated on the hind footpad with CHIKV 181/25 (1.5 10⁵ p.f.u.). This dose was used because lower doses did not produce detectable CHIKV in distal tissues other than the inoculation site in WT mice. As we were focused on early events, comparisons between WT and BST-2−/− mice could not be accomplished with low viral dose. Mice were bled for plasma and PBMCs 24 h later and sacrificed. At necropsy, tissues were collected for viral load determination. When indicated, single cells from lymph node were analysed for surface BST-2 and CD40L expression. Experiments...
involving mice were approved by the University of Iowa Animal Care and Use Committee.

**Cells.** Vero, 293T, HeLa, U937 and MEF cells from WT and BST-2/<sup>−/−</sup> mice [all from the American Type Culture Collection (ATCC)] were maintained in Dulbecco’s modified Eagle’s medium or as recommended by the ATCC.

**Virus stock.** CHIKV strain 181/25 (Levitt et al., 1986) was inoculated onto Vero cells. Upon appearance of cytopathic effects, culture supernatants were harvested, clarified (1000 × g for 5 min), aliquoted and stored (~80 °C) until titres were determined by EPDA on Vero cells.

**Virus replication.** Monolayers of cell in interest in 48-well plates were inoculated with CHIKV (m.o.i. 0.1), allowed to adsorb (1 h at 35 °C), washed, and fresh medium was added to each well. Cells were incubated at 37 °C for 24 h. Infectious fluids were used for titre determination.

**EPDA.** Serial dilutions of infectious fluids were inoculated onto replicate Vero cells. Cells were monitored daily and on day 5 post-inoculation the number of cells infected was determined for each virus dilution by examining cells for cytopathicity. The end-point (day 5 for our assays) was calculated from the data and expressed as TCID<sub>50</sub>. The TCID<sub>50</sub> was expressed as p.f.u. ml<sup>−1</sup> by multiplying the TCID<sub>50</sub> titre by 0.7 (Mills et al., 1971).

**NTA.** NTA is a single-particle detection system that allows measurement of total virus concentration (infectious and non-infectious particles) in liquid suspension. Clarified supernatants from infected cells were injected into an NTA NS300 (NanoSight) sample chamber using a syringe and particle movement was video-captured. For each experiment, mean particle number was counted from 20 frames of videos from measurements per sample recorded over 30 s with a mean error of 2 %. Measurements were taken at room temperature. Data were analysed using NTA 2.3 software. Supernatant virus titre was reported as particles ml<sup>−1</sup>. All buffers were filtered through 0.02 µm filters and checked for absence of particles before use.

**Protein analysis by FACS and ELISA.** Confocal imaging, FACS and ELISA analysis were performed as described previously (Jones et al., 2012, 2013a, b) with antibodies against IFN-γ, BST-2 and CD40L (ebiScience), and appropriate IgG.

**RNA isolation, and viral and host gene mRNA quantification.** RNA was isolated from cells/tissues with the RNeasy Mini kit (Qiagen) and from plasma with the QIAamp Viral RNA kit (Qiagen). Equivalent amounts of RNA treated with DNase I (Qiagen) were reverse transcribed with a high-capacity cDNA reverse transcription kit (ABI) and amplified with target-specific primers (Jones & Okeoma, 2013; Mehta et al., 2012).

**Microscopy.** As we described previously, macrophages plated on coverslips were infected with CHIKV for 24 h. Cells were stained with anti-mouse CHIKV polyclonal antibody, anti-BST-2 antibody and appropriate secondary antibodies (Jones et al., 2013b). Confocal images were acquired using a Zeiss 710 confocal microscope.

**PCR array analysis.** Gene expression was analysed using RT<sup>2</sup> Profiler PCR Array mouse inflammatory cytokines and receptors and RT<sup>2</sup> Real-Time SYBR Green/PCR Master Mix (SA Biosciences). cDNA synthesized from 1 µg total footpad RNA was amplified in the presence of 86 specific primers coated in 96-well microtitre plates on an ABI 7500 Fast Real-Time PCR System. Data were analysed by the web-based Analysis Template provided by SA Biosciences.

**Statistics.** Statistical analysis was performed by paired Student’s t-test.

**ACKNOWLEDGEMENTS**

This work was supported by funds from the Department of Microbiology of the University of Iowa, National Cancer Institute of the National Institutes of Health (P30CA086862) and National Institutes of Health T32 (AI007511). Publication of this paper was made possible through core services from the University of Iowa Central Microscopy Research Facility. We thank Marisa Madison, Alexander Cantfield and Bryson Okeoma of the University of Iowa for help with ELISA, EPDA and constructive criticism, respectively.

**REFERENCES**


