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Marburg Virus Evades Interferon Responses by a Mechanism Distinct from Ebola Virus

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Abstract

Previous studies have demonstrated that Marburg viruses (MARV) and Ebola viruses (EBOV) inhibit interferon (IFN)-α/β signaling but utilize different mechanisms. EBOV inhibits IFN signaling via its VP40 protein which blocks the nuclear accumulation of tyrosine phosphorylated STAT1. In contrast, MARV infection inhibits IFNα/β induced tyrosine phosphorylation of STAT1 and STAT2. MARV infection is now demonstrated to inhibit not only IFNα/β but also IFNγ-induced STAT phosphorylation and to inhibit the IFNα/β and IFNγ-induced tyrosine phosphorylation of upstream Janus (Jak) family kinases. Surprisingly, the MARV matrix protein VP40, not the MARV VP24 protein, has been identified to antagonize Jak and STAT tyrosine phosphorylation, to inhibit IFNα/β or IFNγ-induced gene expression and to inhibit the induction of an antiviral state by IFNα/β. Global loss of STAT and Jak tyrosine phosphorylation in response to both IFNα/β and IFNγ is reminiscent of the phenotype seen in Jak1-null cells. Consistent with this model, MARV infection and MARV VP40 expression also inhibit the Jak1-dependent, IL-6-induced tyrosine phosphorylation of STAT1 and STAT3. Finally, expression of MARV VP40 is able to prevent the tyrosine phosphorylation of Jak1, STAT1, STAT2 or STAT3 which occurs following over-expression of the Jak1 kinase. In contrast, MARV VP40 does not detectably inhibit the tyrosine phosphorylation of STAT2 or Tyk2 when Tyk2 is over-expressed. Mutation of the VP40 late domain, essential for efficient VP40 budding, has no detectable impact on inhibition of IFN signaling. This study shows that MARV inhibits IFN signaling by a mechanism different from that employed by the related EBOV. It identifies a novel function for the MARV VP40 protein and suggests that MARV may globally inhibit Jak1-dependent cytokine signaling.

Introduction

Filoviruses, which include the genera ebolavirus (EBOV) and marburgvirus (MARV), are enveloped negative-strand RNA viruses that cause highly lethal hemorrhagic fever in humans and in non-human primates. The ability of filoviruses to counteract innate antiviral responses of the host, particularly the IFNα/β response is thought to promote uncontrolled virus replication in vivo and thereby contribute to development of severe disease [1]. The IFNs, which include IFNα/β and IFNγ, are antiviral cytokines. IFNα/β are members of a family of proteins that interact with the same ubiquitous receptor to trigger innate antiviral defense mechanisms and promote adaptive immunity [2]. IFNγ also triggers expression of antiviral genes, however, its major function is to modulate adaptive immune responses [3]. IFNα/β signaling results in the tyrosine phosphorylation and activation of the Janus kinases Jak1 and Tyk2. These phosphorylate STAT2 and STAT1, which in turn heterodimerize and associate with interferon regulatory factor 9 (IRF9) to form a complex that is translocated into the nucleus to activate genes involved in antiviral response [reviewed in [4]]. IFNγ signaling activates Jak1 and Jak2, resulting in tyrosine phosphorylation of STAT1. This induces STAT1 homodimerization and translocation to the nucleus such that IFNγ dependent gene expression is induced [reviewed in [4]]. Of note, Jak1, a kinase involved in multiple cytokine signaling pathways, is critical for both IFNα/β and IFNγ signaling. For example, in cells lacking Jak1, IFNα/β fails to trigger STAT1 or STAT2 tyrosine phosphorylation and Tyk2 tyrosine phosphorylation is greatly reduced or eliminated [5,6]. Similarly, in cells lacking Jak1, IFNγ fails to trigger Jak1, Jak2 or STAT1 tyrosine phosphorylation [5,7,8].

Filovirus genomes encode seven structural proteins. Four of these proteins, the nucleoprotein (NP), the viral proteins VP33 and VP30, and the L protein are tightly associated with the RNA genome, form the nucleocapsid and mediate replication and transcription [reviewed in [9]]. Besides its function as polymerase
Author Summary

The closely related members of the filovirus family, Ebola virus (EBOV) and Marburg virus (MARV), cause severe hemorrhagic disease in humans with high fatality rates. Infected individuals exhibit dysregulated immune responses which appear to result from several factors, including virus-mediated impairment of innate immune responses. Previous studies demonstrated that both MARV and EBOV block the type I interferon-induced Jak-STAT signaling pathway. For EBOV, the viral protein VP24 mediates the inhibitory effects by interfering with the nuclear translocation of activated STAT proteins. Here, we show that MARV uses a distinct mechanism to block IFN signaling pathways. Our data revealed that MARV blocks the phosphorylation of Janus kinases and their target STAT proteins in response to type I and type II interferon and interleukin 6. Surprisingly, the observed inhibition is not achieved by the MARV VP24 protein, but by the matrix protein VP40 which also mediates viral budding. Overexpression studies indicate that MARV VP40 globally antagonizes Jak1-dependent signaling. Further, we show that a MARV VP40 mutant defective for budding retains interferon antagonist function. Our results highlight a basic difference between EBOV and MARV, define a new function for MARV VP40 and reveal new targets for the development of anti-MARV therapies.

cofactor, VP35 acts as an inhibitor of antiviral pathways (see below). Two of the filovirus structural proteins are matrix proteins, VP40, the functional equivalent of the matrix (M) proteins of other non-segmented negative-strand RNA viruses, and the minor matrix protein VP24 that is unique to filoviruses. As a peripheral membrane protein VP40 is located at the inner side of the virion membrane. It is critical for viral budding and interacts with cellular proteins involved in vesicle formation to facilitate virus release [10,11,12,13,14,15,16,17,18]. The minor matrix protein VP24 is involved in nucleocapsid formation and assembly [19,20,21,22,23]. EBOV VP42 plays a crucial role in host tropism [24,25] and is able to counteract the type I IFN response (see below). Filoviruses possess a single surface protein, the type I transmembrane glycoprotein GP that mediates attachment to target cells and virus entry. Besides EBOV VP35 and VP24, EBOV GP is the third filoviral protein known to interfere with antiviral cellular functions [26]. Among filoviruses, IFN evasion strategies have been most thoroughly explored for EBOVs. The EBOV species Zaire ebolavirus (ZEOBV) suppresses production of IFNα/β and inhibits cellular responses to IFNα/β and IFNγ [27,28,29,30]. Inhibition of IFNα/β production appears to be mediated by the VP35 protein [31,32], whereas cellular responses to IFNα/β and IFNγ are blocked by the EBOV VP42 protein [33,34]. EBOV VP24 prevents the IFN-induced nuclear accumulation of tyrosine phosphorylated STAT1. This results in inhibition of IFN-induced gene expression and blocks the antiviral effects of IFNs. The inhibition of STAT1 nuclear accumulation is mediated by interaction of VP24 with NPI-1 subfamily of karyopherin α proteins that normally transport dimerized phospho-STAT1 to the nucleus [33,34].

MARVs have a genome organization similar to EBOVs, but they are phylogenetically distinct from EBOVs [35]. Despite their similar genomic organization, morphology and the similarity of MARV versus EBOV induced disease, several biological differences between the viruses have been noted, such as differences in their transcription strategies [36], in the structure of their replication promoters [37], the use of mRNA editing to express the surface glycoprotein by EBOVs but not MARVs [38,39] and differences in the protein requirement for nucleocapsid formation [40,41]. In terms of the capacity of EBOV and MARV to counteract host IFN responses, microarray analyses suggest that ZEOBV and MARV each efficiently suppress host IFN responses, and each virus effectively inhibits cellular responses to exogenously added IFNα [30]. However, examination of the phosphorylation status of STAT1 following addition of IFNα to infected cells revealed an intriguing difference between ZEOBV and MARV. While ZEOBV did not inhibit the IFNα-induced tyrosine phosphorylation of STAT1, MARV infection resulted in an inhibition of both STAT1 and STAT2 tyrosine phosphorylation [30].

The present study demonstrates that MARV infection inhibits not only IFNα/β but also IFNγ and Jak1-dependent IL-6 signaling. Further, the MARV protein mediating these effects has been identified. We show that expression of the MARV matrix protein VP40 is sufficient to block IFN and IL-6 signaling pathways. Experiments in which either Jak1 or Tyk2 are overexpressed suggest that MARV VP40 targets Jak1 function. These observations identify an important difference in the biology of MARV and EBOVs, identify a novel function for a negative-strand virus matrix protein and suggest that MARV may inhibit multiple Jak1-dependent cytokine signaling pathways.

Results

MARV infection prevents IFN-mediated phosphorylation and nuclear translocation of STAT proteins

Previous studies demonstrated that tyrosine phosphorylation of STAT1 and STAT2 is strongly reduced in MARV- but not in ZEOBV-infected Huh-7 cells treated with IFNα [30]. To confirm this observation and to determine whether MARV inhibition extends to other Jak-STAT signaling pathways, the impact of MARV infection on IFNα-induced STAT1 and STAT2 phosphorylation and on IFNγ-induced STAT1 phosphorylation was compared. As reported, MARV but not EBOV inhibited phosphorylation of endogenous STAT1 and STAT2 induced by IFNα (Fig. 1A). MARV also inhibited IFNγ-induced STAT1 phosphorylation, whereas EBOV did not (Fig. 1B). For these studies, immunofluorescence analyses were performed in parallel to confirm that more than 95% of cells were infected with either virus (data not shown). These data show that MARV not only blocks type I but also type II IFN signaling by interfering with an early step of the Jak-STAT signaling cascade.

Since previous studies indicated that the nuclear translocation of phosphorylated STAT1 is inhibited in EBOV-infected cells [33,34], we examined the cellular localization of STAT1 in MARV-infected cells by immunofluorescence (Fig. 1C). As expected, STAT1 was translocated into the nucleus in non-infected cells treated with IFNα (left panel, red staining), whereas IFNα-induced translocation was inhibited in ZEOBV-infected cells (right panels, infected cells shown in green). Please note that a single non-infected cell in the ZEOBV infection panel showed nuclear accumulation of STAT1. Nuclear translocation of STAT1 was also blocked in MARV-infected cells treated with IFNα (middle panels).

Taken together, these results highlight a fundamental difference in the mechanisms by which MARV and EBOV counteract innate immune responses.

IFNα-induced tyrosine phosphorylation of Janus kinases is inhibited in MARV-infected cells

Since our data suggested that MARV infection leads to the inhibition of IFN-induced STAT phosphorylation, we next sought
to determine the activation status of Jak1 and Tyk2, the Janus kinases involved in IFNα-induced phosphorylation of STAT proteins. Huh-7 cells were infected with MARV or ZEBOV, treated with IFNα, and the phosphorylation state of endogenous Jak1 and Tyk2 was analyzed by western blot analysis. As shown in Figure 2A, both kinases were phosphorylated in ZEBOV-infected cells in response to IFNα, although phosphorylation of Jak1 was less pronounced compared to non-infected cells (Fig. 2A, compare lane 2 and 4). However, only background levels of Jak1 phosphorylation were detectable and Tyk2 phosphorylation was completely blocked in MARV-infected cells treated with IFNα (Fig. 2A, lane 6). From this we concluded that the inhibition of the Jak-STAT-signaling pathway by MARV takes place upstream of Jak phosphorylation or directly at the Janus kinases.

As part of the innate immune response, the Jak-STAT signaling cascade acts as a first line of defense to prevent viral infections. Therefore, we determined at which time point of the MARV replication cycle the observed inhibition of Jak activation occurs. Further, we asked whether live virus and viral replication are needed to antagonize IFN signaling. Huh-7 cells were infected with live MARV or UV-inactivated MARV, treated with IFNα, harvested at different time points post-infection (p.i.) and subjected to western blot analysis to determine the phosphorylation state of Tyk2. While Tyk2 was still efficiently phosphorylated in MARV-infected and IFN-treated cells at 1 hour and 2.5 hours p.i., respectively, near complete inhibition of Tyk2 phosphorylation was achieved at 4 hours p.i. (Fig. 2B). A single MARV replication cycle takes approximately 21 hours in Vero E6 cells [42]. Thus, it can be concluded that the observed antagonistic effect occurs early in infection. Additionally, since MARV infection did not lead to the inhibition of Tyk2 phosphorylation at time points earlier than 4 hours p.i., it is assumed that binding of MARV to its receptor does not trigger its IFN antagonist function. Interestingly, infection of cells with UV-inactivated MARV prior to IFNα treatment did not lead to the inhibition of Tyk2 phosphorylation (Fig. 2B), supporting the assumption that receptor binding does not play a role in the MARV-specific inhibition of the IFN signaling cascades. In addition, these data indicate that intracellular virus replication is required for the observed antagonistic effects.

To examine whether MARV indirectly inhibits Jak1 phosphorylation via protein tyrosine phosphatases (PTPs), we treated MARV-infected and IFN-treated cells with different PTP inhibitors prior to IFNα stimulation. Besides an inhibitor against PTP1B, which specifically dephosphorylates Tyk2 and Jak2 [43], we tested the broad acting phosphatase inhibitor sodium orthovanadate. Our results show that even in the presence of PTP inhibitors Tyk2 phosphorylation was inhibited in MARV-infected cells (Fig. 2C), suggesting that the observed inhibitory effects do not depend on active cellular PTPs.

Figure 1. MARV infection prevents IFN-mediated phosphorylation and nuclear translocation of STAT proteins. Huh-7 cells were infected with MARV or ZEBOV at an MOI of 5 or mock-infected (Mock). At 24 h p.i., cells were either treated with 100 IU/ml of IFNα-2b (A) or 10 IU/ml of IFNγ (B) for 30 min where indicated. Cell lysates were analyzed by western blotting using antibodies directed against total and phosphorylated forms of STAT1 (A and B) or STAT2 (A). (C) Huh-7 cells were infected with MARV or ZEBOV at an MOI of 5 or left uninfected. At 24 h p.i., the cells were stimulated with 1000 IU/ml of IFNα-2b for 45 min, fixed with 4% paraformaldehyde, and stained with antibody directed against STAT1, MARV, or ZEBOV as indicated.

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MARV matrix protein VP40 acts as an IFN antagonist

To identify the viral protein mediating the antagonistic effects observed in MARV-infected cells, individual EBOV or MARV proteins were assessed for their capacity to counteract the antiviral effects of IFNβ (Fig. 3A). Vero cells were transfected with expression plasmids; one day post-transfection the cells were either mock-treated or treated overnight with IFNβ, and the cells were then infected with a Newcastle disease virus that expresses GFP (NDV-GFP). Since NDV is IFN-sensitive, GFP expression in these cells provides a measure of virus replication, and suppression of GFP expression provides a read-out for the antiviral effects of IFNβ. While empty vector (pCAGGS)-transfected, mock-treated cells permitted NDV-GFP replication, IFNβ-treated, empty vector-transfected cells, in contrast, greatly suppressed GFP expression (Fig. 3A, panels 1 and 2). As previously described, expression of Nipah virus W protein, or ZEBOV VP24, known inhibitors of IFN signaling, rescued replication of NDV-GFP in IFNβ-treated cells [33,44] (Fig. 3A, panel 3 and 4). Surprisingly, MARV VP24 did not detectably counteract the antiviral effects of IFNβ (Fig. 3A, panel 6). In fact, the only MARV protein tested that clearly permitted NDV-GFP replication in IFNβ-treated cells was the major matrix protein VP40 (Fig. 3A, panel 9). In contrast, the homologous ZEBOV protein, ZEBOV VP40, did not support NDV-GFP replication (Fig. 3A, panel 5).

To confirm the finding that MARV VP40 antagonizes IFN signaling, we analyzed the intracellular distribution of STAT2 in cells transiently expressing MARV or EBOV proteins VP35, VP24, or VP40 (Fig. 3B). Since it has been shown by Brzoza et al. [45] that rabies virus phosphoprotein (P) efficiently blocks the nuclear translocation of STAT2 into the nucleus, P was used as a positive control (Fig. 3B). Cells transfected with empty vector served as a negative control. While expression of either MARV VP40 or ZEBOV VP24 led to a significant inhibition of STAT2 accumulation in the nucleus, none of the other tested filoviral
Figure 3. MARV VP40 acts as an IFN antagonist. (A) Vero cells were transfected with 1 µg empty vector (pCAGGS) or the indicated expression plasmids. 24 h post transfection (p.t.) cells were treated with 1000 IU/ml of IFNβ for 24 h and infected with NDV GFP. At 16 h p.i., green fluorescence (indicating viral replication) was visualized and photographed with a fluorescence microscope. (B) Huh-7 cells were transfected with 2 µg of the indicated plasmids expressing the indicated viral proteins from rabies virus, ZEBOV and MARV. At 24 h p.t., the cells were stimulated with 2000 IU/ml of IFNα-2b for 45 min, fixed with 4% paraformaldehyde, and stained with anti-STAT2 antibody and antibodies to detect viral proteins. doi:10.1371/journal.ppat.1000721.g003
proteins including ZEBOV VP40 and MARV VP24 was able to inhibit nuclear translocation of STAT2 in response to IFNγ (Fig. 3B). From this, we concluded that MARV VP40 is the viral protein interfering with IFN signaling.

**MARV VP40 inhibits type I and type II IFN-induced STAT and Jak activation**

Our results obtained with infected cells clearly show that MARV infection leads to the inhibition of STAT and Jak phosphorylation, whereas ZEBOV infection does not. To assess the impact of MARV VP40 on IFN-induced signaling in the absence of other viral proteins, STAT1-GFP or STAT2-GFP were co-transfected into Huh-7 cells with empty vector or with plasmids expressing ZEBOV VP40, ZEBOV VP24, MARV VP40 or MARV VP24. The phosphorylation state of the STAT proteins in response to IFNα/β (Fig. 4A) and IFNγ (Fig. 4B) was examined by western blot analysis. Expression of the Langat virus NS5 protein (LGTV NS5), a protein previously demonstrated to inhibit STAT1 and STAT2 tyrosine phosphorylation [16] served as a control. Following addition of IFNα/β to transfected Huh-7 cells, MARV VP40 inhibited the IFNα/β-induced tyrosine phosphorylation of either STAT1-GFP (Fig. 4A, left panel) or STAT2-GFP (Fig. 4A, right panel). In contrast, the ZEBOV VP40, ZEBOV VP24 and MARV VP24 proteins failed to inhibit STAT1 or STAT2 tyrosine phosphorylation (Fig. 4A). Relative to empty vector-transfected cells, LGTV NS5 reduced the IFNγ-induced phosphorylation of STAT1-GFP (Fig. 4B), but ZEBOV VP24, MARV VP24 and ZEBOV VP40 failed to inhibit STAT1 phosphorylation. In contrast, MARV VP40 expression led to a substantial reduction in IFNγ-induced STAT1 tyrosine phosphorylation (Fig. 4B).

Next, we analyzed the impact of MARV VP40 on the phosphorylation of Janus kinases in cells treated with IFNα/β or IFNγ. 293T cells were transfected with empty vector or plasmids that express LGTV NS5, ZEBOV VP24, ZEBOV VP40, MARV VP24 or MARV VP40, treated with IFNα/β and analyzed for phosphorylation of endogenous Jak1 and Tyk2. MARV VP40 inhibited the IFNα/β-induced tyrosine phosphorylation of both kinases (Fig. 4C). Interestingly, none of the other expressed proteins including LGTV NS5 detectably blocked Jak1 phosphorylation. Although Tyk2 phosphorylation was also reduced by LGTV NS5 and to a lesser extent by ZEBOV VP24, this reduction was less pronounced compared to cells expressing MARV VP40 (Fig. 4C). Similar results were obtained in cells treated with IFNγ. Inhibition of Jak1 and Jak2 phosphorylation in response to IFNγ treatment was only observed in cells expressing MARV VP40 (Fig. 4D). Taken together, these results clearly confirm that MARV not only uses a different mechanism than ZEBOV to block IFN signaling, but an alternate viral protein carries out this function.

**MARV VP40 inhibits ISRE- and GAS-induced gene expression**

To address the functional significance of the observed inhibition, the impact of MARV VP40 on IFNβ and IFNγ-induced transcription was assessed by reporter gene assay (Fig. 5). Two reporter constructs were used. One, activated by IFNα/β, possesses an ISG54 promoter and contains an interferon stimulated response element (ISRE). The second, activated by IFNγ, possesses three gamma activated sequence (GAS) elements. 293T cells were transfected with either reporter plus expression plasmids for MARV VP40 or, as controls, MARV VP24 and ZEBOV VP24. To control for non-specific or cytotoxic effects of the viral proteins, the results of these assays were normalized to a co-transfected constitutively-expressed Renilla luciferase reporter plasmid. MARV VP40 and ZEBOV VP24 inhibited ISG54 promoter activation, whereas MARV VP24 failed to inhibit its activation (Fig. 5A). Similarly, MARV VP40 inhibited IFNγ-induced gene expression, consistent with its capacity to block IFNγ activation of STAT1. As previously described, ZEBOV VP24 inhibited IFNγ-induced gene expression [33], but MARV VP24 did not inhibit gene expression in this assay (Fig. 5B).

The impact of MARV VP40 upon IFNγ-induced production of the 10 kDa interferon-gamma-induced protein (IP-10), an immune cell chemotaxtractant protein secreted by several cell types in response to IFNγ, was also assessed. Human umbilical vein endothelial cells (HUVECs) were transfected with the indicated expression plasmids, treated with IFNγ, and cell supernatants were tested for the presence of IP-10 by ELISA. MARV VP40 and, to a lesser extent, ZEBOV VP24 inhibited IP-10 expression, whereas MARV VP24 did not (Fig. 5C). To assess the specificity of this effect and exclude cell death or disruption of membrane signaling components, a similar assay was performed testing the impact of viral protein expression on TNFα-induced secretion of IL-8 which is NF-κB-mediated [47,48]. None of the expressed proteins, including MARV VP40, detectably affected IL-8 production (Fig. 5D). Therefore the impact of MARV VP40 seems to be specific for Jak-STAT signaling and does not extend to the induction of NF-κB-mediated signaling [47].

**MARV infection and expression of MARV VP40 inhibit IL-6-induced STAT1 and STAT3 phosphorylation**

Interestingly, our observations are reminiscent of the phenotype seen in Jak1-deficient cells, where the absence of Jak1 results in loss of Jak1, Tyk2, STAT1 and STAT2 tyrosine phosphorylation in response to IFNα/β and loss of Jak1, Jak2 and STAT1 tyrosine phosphorylation in response to IFNγ [5,7,8]. To examine whether the observed inhibitory effect of MARV on IFN signaling extends to other, non-IFN, Jak-STAT signaling pathways, we next analyzed the IL-6-induced activation of STAT1 and STAT3 in MARV-infected cells and cells expressing VP40. IL-6 was chosen because, in Jak1-deficient cells, IL-6-induced STAT1 phosphorylation was absent, and STAT3 phosphorylation was greatly reduced [7]. Huh-7 cells were infected with MARV, treated with IL-6 at 24 hours p.i. and cell lysates were subjected to western blot analysis. As shown in Figure 6A, phosphorylation of endogenous Jak1-deficient cells, IL-6-induced STAT1 phosphorylation was not detectable and STAT3 phosphorylation was greatly diminished in MARV-infected, IL-6 treated cells, reflecting the phenotype of Jak1-deficient cells [7]. Similar results were obtained with transfected Huh-7 cells. MARV VP40 inhibited the IL-6 induced tyrosine phosphorylation of STAT1-GFP to undetectable levels, and FLAG-STAT3 tyrosine phosphorylation was highly reduced (Fig. 6B and C). In contrast, ZEBOV VP40, ZEBOV VP24 and MARV VP24 did not inhibit phosphorylation of either STAT1 or STAT3 (Fig. 6B and C).

**MARV VP40 inhibits phosphorylation of over-expressed Jak1**

To further assess the capacity of MARV VP40 to target the function of Jak1, MARV VP40, ZEBOV VP40, MARV VP24 or ZEBOV VP24 were co-transfected with expression plasmids for STAT2-GFP and either HA-tagged Jak1 or HA-tagged Tyk2. Over-expression of Janus kinases leads to their tyrosine phosphorylation [49] and to the phosphorylation of STAT proteins [Fig. 7].

First, we determined the phosphorylation state of HA-Jak1, MARV VP40, ZEBOV VP40, MARV VP24 or ZEBOV VP24 and either HA-tagged Jak1 or HA-tagged Tyk2. Over-expression of Janus kinases leads to their tyrosine phosphorylation [49] and to the phosphorylation of STAT proteins [Fig. 7]. First, we determined the phosphorylation state of HA-Jak1 and STAT2-GFP in transfected cells by western blot analysis (Fig. 7A). While MARV VP40 completely inhibited the phosphorylation of
Figure 4. MARV VP40 inhibits IFN-induced STAT and Jak phosphorylation. (A) STAT1 or STAT2 (1 μg) fused to a C-terminal GFP was co-expressed in Huh-7 cells with 2 μg of the indicated expression plasmids, treated with 1000 IU/ml of universal IFNα/β for 30 min. Cells were lysed and assayed by western blot for tyrosine phosphorylated STAT1 (p-STAT1-GFP), STAT2 (p-STAT2-GFP), as well as for total expression levels of over-expressed proteins. (B) Huh-7 cells were transfected with 2 μg of the indicated expression plasmids and 1 μg of a plasmid expressing STAT1 fused to GFP at the C-terminus (STAT1-GFP). 24h p.t., cells were treated with 1000 IU/ml of IFNγ for 30 min and lysed. Lysates were analyzed by western blot for phosphorylation of STAT1 and total levels of STAT1 as well as for expression of the tagged proteins. (C and D) 293T cells were transfected with 2 μg of the indicated expression plasmids, treated with 1000 IU/ml of IFNα/β (C) or IFNγ (D) for 30 min, lysed and subjected to western blot analysis for detection of phosphorylated and total Jak1 (C and D), Tyk2 (C) or Jak2 (D). Note that the cuts in the films excised samples irrelevant to this study. All the presented data for a given protein is from the same gel and the same exposure.

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over-expressed HA-Jak1 and consequently, the phosphorylation of STAT2-GFP, ZEBOV VP40, ZEBOV VP24 and MARV VP24 did not show any inhibitory effect (Fig. 7A). Extending this observation, HA-Jak1 over-expression also led to tyrosine phosphorylation of endogenous STAT1 and STAT3, and this was inhibited by MARV VP40 but not by the other tested viral
proteins (Fig. 7B). In contrast, none of the expressed filovirus proteins, including MARV VP40, detectably inhibited tyrosine phosphorylation of over-expressed HA-Tyk2 or Tyk2-induced STAT2-GFP phosphorylation (Fig. 7C). Further titration of HA-Tyk2 expression was performed, and phosphorylation of endogenous STAT1 was monitored (Fig. 7D). Two-fold dilutions of HA-Tyk2 plasmid were transfected with either empty vector or MARV VP40 plasmid. When 500 ng of Tyk2 plasmid was transfected, less phospho-Tyk2 was detected in the MARV VP40-expressing cells than in cells receiving empty vector. Similarly, levels of phosphorylated endogenous STAT1 were decreased in the presence of MARV VP40. However, the total levels of HA-Tyk2 were also decreased in the presence of MARV VP40 in these samples (Fig. 7D). Therefore the bands were quantified by densitometry and the ratio of phospho-Tyk2 to total Tyk2 was calculated for each sample. In all samples the ratios were in the range of 0.85 to 1.05, suggesting that the decreased levels of phospho-Tyk2 were due to reduced total levels of Tyk2 (data not shown). These data support a model where MARV VP40 targets Jak1 function but do not completely exclude the possibility that MARV VP40 has a modest capacity to inhibit Tyk2.

MARV VP40 inhibition of Jak1-dependent signaling does not require an intact late domain

MARV VP40 contains a late domain (PPPY), positioned from residues 16–19, that mediates VP40 interaction with the cellular protein Tsg101, a component of the ESCRT I machinery, and contributes to its budding function [18]. To determine whether this late domain is critical for MARV VP40 inhibition of signaling, the 16-PPPY-19 motif was mutated to 16-AAAA-19 (M40-AAAA). Relative to wild-type EBOV VP40 or wild-type MARV VP40, M40-AAAA exhibited greatly reduced budding, in the form of virus-like particles (VLPs), from transfected 293T cells, despite comparable expression in the whole cell extracts (Fig. 8A). As expected, a separately expressed GFP was not released into the cell medium (Fig. 8A). When tested for its ability to suppress IFNα/β-induced signaling, the mutant suppressed STAT1 phosphorylation comparably to either LGTV NS5 or wild-type MARV VP40.
Figure 7. Jak1 phosphorylation is inhibited by MARV VP40. (A and C) 1 μg of HA-tagged human Jak1 (A) or Tyk2 (C) expression plasmid was transfected into Huh-7 cells along with 2 μg of empty vector (pCAGGS) or the indicated viral protein expression plasmids and 0.5 μg plasmid encoding STAT2 fused to GFP. Cells were lysed and subjected to western blot analysis using total and phospho-specific antibodies against Jak1 (A), Tyk2 (C), and STAT2 or GFP. Anti-β-tubulin was used as a loading control and anti-Flag to detect expression of viral proteins. (B) 1 μg of human Jak1 plasmid was transfected along with 2 μg of empty vector (pCAGGS) or the indicated viral protein expression plasmids. Cells were lysed, subjected to SDS-PAGE and analyzed with total and phospho-specific antibodies against Jak1, STAT1 and STAT3. Anti-β-tubulin was used as a loading control and anti-Flag to detect expression of viral proteins. (D) Two-fold dilutions of HA-Tyk2 expression plasmid starting at 1 μg were transfected in Huh-7 cells with either 2 μg of empty plasmid (pCAGGS) or 2 μg of expression plasmid for MARV VP40. Levels of phospho-Tyk2 and phospho-STAT1 as well as total Tyk2 and STAT1 were assayed by western blot. Anti-β-tubulin and anti-Flag antibodies were used as indicated. Note that for panels A and B, the cuts in the films excised samples irrelevant to this study. All the presented data for a given protein is from the same gel and the same exposure. doi:10.1371/journal.ppat.1000721.g007
Figure 8. **MARV VP40 inhibition of IFN signaling does not require an intact late domain.** (A) M40-AAAA buds less efficiently than wild type MARV VP40. A MARV VP40 late domain mutant (M40-AAAA) was made using site directed mutagenesis. M40-AAAA, wild type MARV VP40, ZEBOV VP40 or GFP (2 \(\mu\)g) was expressed in 293T cells. 48 h later, supernatants were harvested and virus-like particles (VLPs) were purified through a sucrose cushion. Cells were lysed and examined together with the VLPs for protein expression levels (WCE: whole cell extract). (B) MARV VP40 does not require the late domain to inhibit the phosphorylation of STAT1. Huh-7 cells were transfected with 1 \(\mu\)g STAT1-GFP expression plasmid along with 2 \(\mu\)g plasmid DNA encoding Flag-tagged versions of viral proteins from LGTV, ZEBOV and MARV or the late domain mutant M40-AAAA and treated with 1000 IU/ml of IFN-\(\alpha/\beta\) for 30 min. Lysates were analyzed for phosphorylation of STAT1 and for total expression levels of all over-expressed proteins. \(\beta\)-tubulin expression was assessed as indicated. All the presented data for a given protein is from the same gel and the same exposure. (C) MARV VP40 does not need the late domain to inhibit the IFN-\(\alpha/\beta\)-dependent induction of ISG54-Luc. 293T cells were co-transfected with a construct expressing the luciferase gene under control of an ISG54 promoter along with a constitutively expressed Renilla luciferase reporter gene and 1 \(\mu\)g empty vector (pcAGGS) or expression plasmids expressing wild-type MARV VP40 or M40-AAAA. Cells were treated with 1000 IU/ml IFN-\(\alpha/\beta\) for 18 h and assayed for firefly and Renilla luciferase activities. IFN-induced firefly luciferase activity was normalized to Renilla luciferase activity. The bars represent the mean fold induction of 3 experiments compared to the untreated negative control and error bars represent the standard deviations. doi:10.1371/journal.ppat.1000721.g008
Towner and colleagues reported the successful isolation of MARV yet Ebola viruses have not been isolated from bats. In contrast, seropositive or RT-PCR-positive for EBOV [52,53], however, as of filoviruses. Various species of African fruit bats were found to be only limited information available about the natural host spectrum account for different immune evasion mechanisms. So far, there is thousands of years ago (S.T. Nichol, personal communication). Analysis of genome sequence differences indicates that Ebola and evolutionary paths taken by Marburg and Ebola viruses. Bayesian to block IFN signaling may have been driven by the different VP40 [56].

Host tropism has yet to be determined; however, it is intriguing ZEBOV [24,25]. Whether VP40 plays a similar role in MARV Asian species. Notably, VP24 contributes to the host specificity of among Ebola viruses prior to the separation of the African and species is also discussed as a potential reservoir for EBOV [53], it remains unclear if Marburg and Ebola viruses differ in their host tropism. Recently, the Asian EBOV species remains to be determined if these proteins that suppress IFN-β appears to serve the same purpose as the EBOV VP24 proteins which also counteract IFN-β signaling. It is likely that counteracting IFN-β signaling has a significant impact on viral pathogenesis in vivo, because, despite the presence of viral VP35 proteins that suppress IFN-β/production [31,58,59,60], filovirus replication in vivo results in significant IFN-β production [61]. The presence of IFN-β/ signaling inhibitors likely also contributes to the relative insensitivity of filoviruses to IFN-β/ as an antiviral therapy [50]. IFN-γ also has antiviral properties [62], however, suppression of IFN-γ signaling may also modulate adaptive immune responses to infection. For example, human cytomegalovirus down-regulates Jak1 expression in a proteasome-dependent manner, and although a specific viral gene product that mediates this effect has not been defined, this function prevents the IFN-γ-induced upregulation of MHC class II on infected cells [63]. Another viral protein that interacts with Jak1 and blocks the type I IFN signaling pathway is the measles virus V protein, but the consequence of this function for adaptive immunity has not been defined [64]. The possible impact of MARV infection and MARV VP40 expression on other cytokine signaling pathways involving Jak1 remains to be defined. Given the prominent role of Jak1 in numerous pathways, the impact of MARV VP40 on cytokine signaling may be quite broad.

Filovirus VP40 proteins are matrix proteins sufficient to drive budding of virus-like particles, and they are thought to be the driving force for the budding of infectious virus [11,13,18,65,66,67]. The finding that MARV VP40 also serves as an inhibitor of IFN signaling is surprising and novel. Another example of a negative-strand RNA virus matrix protein that inhibits IFN responses is the vesicular stomatitis virus (VSV) matrix protein (M). VSV M inhibits innate immune responses, including IFNβ production, by a mechanism different from MARV VP40, inhibiting host cell transcription as well as nucleo-cytoplasmic transport of cellular mRNAs [68,69,70,71].
Host factors that interact with filovirus VP40 proteins have been described [14,18,65,72,73]. The most fully characterized interactions occur via the VP40 late domain which facilitates budding and release of virus particles. ZEBOV VP40 possesses two late domains, a PTAP motif and an overlapping PXXP motif [11,65]. These mediate interaction with Tsg101, Nedd4, and Rsp5 [14,65]. MARV VP40 possesses a single PPPY motif that allows interaction with Tsg101 [18]. To address the potential role of these well-characterized motifs in MARV VP40 inhibition of Jak/STAT signaling, a 16-PPPY-19 to 16-AAAA-19 mutant MARV VP40 was generated. As previously described, this mutation severely impaired MARV VP40 budding (Fig. 8A) [18]. Yet this mutation had no detectable impact on MARV VP40 inhibition of IFN/β signaling (Fig. 8B and C). Therefore, the late domain is dispensable for the IFN signaling function of VP40, and the budding and signaling functions of MARV VP40 appear to be separable. Of note, IFN-induced cellular inhibitors of filovirus VP40 budding have recently been described. These include the IFN stimulated ISG15 and tetherin [26,74,75]. ISG15 is an IFN-induced protein which inhibits budding of EBOV VP40. ISG15 inhibits the ubiquitin ligase Nedd4, which interacts with EBOV VP40 through the PPXY motif to promote VP40 ubiquitination and budding [65,75,76]. Tetherin is constitutively-expressed in some cell types but is also IFN-inducible. Its expression can prevent release of VLPs produced following expression of EBOV or MARV VP40 [26,74,77]. Co-expression of EBOV GP has been shown capable of counteracting this antiviral function [26]. Whether MARV GP can also inhibit tetherin has not yet been addressed; however, because MARV VP40 can inhibit IFN signaling, it appears to have a built-in capacity to resist IFN-induced mechanisms that target viral budding.

This study has identified an important difference in the biology of MARV and EBOV, defined a novel function for the MARV VP40 matrix protein and suggests that MARV may inhibit multiple Jak1-dependent cytokine signaling pathways. Future studies will determine whether the different means by which EBOV and MARV counteract cell signaling pathways result in significant differences in the pathogenesis of these viruses. Determining the molecular mechanisms by which MARV VP40 blocks signaling may facilitate development of new anti-MARV therapies.

Materials and Methods

Cell lines and viruses
293T, Vero E6, Vero (ATCC, Manassas, VA) and Huh-7 (kindly provided by Dr. DiFeo, Mount Sinai School of Medicine) cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 10 mM HEPES pH 7.5 or in DMEM supplemented with penicillin (50 units/ml), streptomycin (50 mg/ml) and 10% fetal bovine serum. HUVECs were maintained in F-12K medium (kindly provided by Dr. DiFeo, Mount Sinai School of Medicine) supplemented with 0.1 mg/ml heparin (Sigma-Aldrich, St. Louis, MO), 0.03 mg/ml endothelial cell growth supplement (ECGS) (Sigma-Aldrich), and 10% fetal bovine serum (HyClone). A previously-described Newcastle disease virus engineered to express green fluorescence protein (NDV-GFP) was propagated in 10-day-old embryonated chicken eggs [77]. ZEBOV strain Mayinga and MARV strain Musoke were grown and propagated as described previously [39]. All work with infectious filoviruses was performed under biosafety level 4 conditions at the Institute of Virology, Philipps University of Marburg, Marburg, Germany.

Plasmids
PCR products corresponding to FLAG-tagged, HA-tagged or untagged viral proteins of EBOV (Accession # NC002349) and MARV (Accession # NC016006) were cloned into the pcAGGS or pcDNA3.1 expression vectors [78]. The Nipah Virus W (NV W) protein expression plasmid was previously described [77]. The expression plasmid for V3-tagged Langat Virus NS5 was previously described [46]. Human Jak1 (Accession # BAAE2826) and Tyk2 (Accession # NP_003322) were RT-PCR amplified from RNA isolated from 293T cells and cloned with an HA tag into the pcAGGS vector. For the generation of the late domain mutants, site directed mutagenesis was performed using the QuickChange II kit (Stratagene, La Jolla, CA). A Flag-tagged Rabies P expression plasmid in a pCR3 background was kindly provided by Drs. Conzelmann and Brzozka (Ludwig Maximilian University, Munich, Germany).

Transfections
293T cells were transfected using Lipofectamine 2000 (LP2K) at a ratio 1:1 with plasmid DNA (µg DNA: µL LP2K). Vero cells were transfected using LP2K at a ratio 1:2. Huh-7 cells were transfected using LP2K at a ratio 1:2.75. HUVEC cells were electroporated using the AMAXA nucleofector II, nucleofection program V-001 and solution V according to the manufacturer’s directions (Lonza, Walkersville, MD). Cells were lysed with an IGEPAL lysis buffer (50 mM Tris [pH 8.0], 280 mM NaCl, 0.3% IGEPAL, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol (DTT) supplemented with protease inhibitor cocktail (Roche) and 0.1 mM Na2VO4) [79] for 30 min on ice and spun at 13KRP on a refrigerated tabletop centrifuge for 1 minute.

Cytokines
Universal type I IFN (a consensus IFNα/β), human IFNβ and human IFNγ (PBL, Piscataway, NJ) were used at 1000 IU/ml unless otherwise specified for 30 min in RPMI (GIBCO) or phosphate buffered saline (PBS) supplemented with 0.3% BSA. Human IFNα-2b (Essex Pharma, Kenilworth, NJ) diluted in PBS supplemented with 0.1% BSA was used at 1000–2000 IU/ml unless otherwise specified. Human TNFα (Peprotech, Rocky Hill, NJ) was used at 50 ng/ml for 24 hours in HUVEC culture medium as described above. Human IL-6 (Peprotech, Rocky Hill, NJ) was used at 50 ng/ml in RPMI supplemented with 0.3% BSA.

Inhibition of IFNβ-induced antiviral state
4×10⁵ Vero cells per well were cultured in 24 well plates and transfected with 1 µg of each plasmid encoding viral proteins. At 24 hours post-transfection cells were treated with IFNβ (1000 IU/ml) for 24 hours. Then cells were infected with 5 haemagglutinating units of NDV-GFP virus in a volume of 200 µl of 0.3% BSA in PBS for 1 h, washed twice and replaced with DMEM supplemented with 10% FBS. GFP expression was visualized at 16 hours post-infection with a fluorescence microscope.

Reporter gene assays
293T cells (5×10⁵) or Huh-7 (3×10⁵) were transfected with 0.5 µg of a construct having an IFN-stimulated gene 54 promoter driving expression of a chloramphenicol acetyltransferase (CAT) reporter gene (pISG54-CAT), 0.1 µg of a constitutively expressing Renilla luciferase reporter construct (pCAGGS-luc), and the indicated amounts of the expression plasmids. Twenty-four hours post-transfection, cells were washed and treated with IFN (as described above). Sixteen hours post-IFN treatment, cells were harvested using reporter lysis buffer (Promega, Madison, WI) and
analyzed for CAT and luciferase activities by standard methods. CAT activity was quantified by using a PhosphorImager and normalized to the luciferase activity. Alternatively, an ISG54-firefly luciferase reporter plasmid (pISG54-Luc) (0.3 μg) reporter was used, and a dual luciferase reporter (DLR) assay was performed according to the manufacturer’s guidelines (Promega). For IFNγ-dependent gene expression, a reporter having 3 copies of the gamma activated sequence driving the expression of firefly luciferase (GAS-Luc) (0.3 μg) was transfected with 0.1 μg of a constitutively expressing luciferase reporter construct (pCAGGS-luc), and the indicated amounts of the expression plasmids. Twenty-four hours post-transfection, cells were washed and treated with IFNγ (as described above). Sixteen hours post-IFN treatment cells were harvested and analyzed using a DLR assay (Promega). Assays were performed in triplicate and p-values were calculated by a two-tailed Student’s t-test for unpaired samples using the software GraphPad Prism (GraphPad Software, Inc.).

Western blot analysis of transfected cells and ELISAs

For the detection of the overexpressed viral proteins, the anti-V5 (Invitrogen), anti-HA and anti-Flag M2 (Sigma) antibodies were used at a 1:5000 dilution in 1% non-fat dry milk in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4; 150 mM NaCl). As a loading control, anti-beta tubulin (Sigma) antibody was used at a 1:10,000 dilution in 1% non-fat dry milk in TBS. Anti-GFP was used at a 1:10,000 dilution in 1% non-fat dry milk in TBS (Clontech, Mountain View, CA). Phosphorylated STAT1 was detected with a phospho-tyrosine specific antibody recognizing phospho-Y701 (BD Transduction Laboratories, San Jose, CA), and total levels of STAT1 with an antibody recognizing the STAT1 C-terminus (BD Transduction Laboratories) diluted to 1:1000 and 1:500, respectively, in 1% non-fat dry milk in TBS. STAT2 and its phosphorylated form (pY689) were detected with polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA and Upstate, Lake Placid, NY respectively) diluted 1:500 in 1% non-fat dry milk in TBS. STAT3, pY705-STAT3, Tyk2, pY1054/1055-Tyk2, pY1022/1023-Jak1, pY1007/1008-Jak2 (Cell Signalling, Beverly, MA), Jak1 (BD Transduction Laboratories) and Jak2 (Millipore, Billerica, MA) were used at a 1:50 dilution in TBS, 0.1% Tween and 5% BSA.

For the detection of IP-10 and IL-8, supernatants of transfected HUVECs treated with 100 IU/ml human IFNγ or 50 ng/ml TNFα for 24 hours were collected and diluted 1:100 and 1:1000, respectively, in PBS supplemented with 5% fetal bovine serum. The BD OptEIA Human IP-10 and Human IL-8 kits were used (BD Biosciences, Franklin Lakes, NJ).

Western blot analysis of filovirus-infected cells

Huh-7 cells grown on glass coverslips were infected with ZEBOV or MARV at an MOI of 5 or left uninfected. At 24 hours p.i., cells were washed twice with PBS and inactivated by treatment with 4% paraformaldehyde for 24 hours. Cells were then permeabilized with a mixture of acetone and methanol (1:1, vol/vol) for 5 min at −20°C and treated with 0.1 M glycine. As primary antibodies, a rabbit antiserum directed against the nucleocapsid complex of MARV (1:100) and a goat antiserum directed against ZEBOV (1:500) (kindly provided by Dr. Becker, Philipps University of Marburg, Marburg, Germany) were used. To detect endogenous STAT1 or STAT2 proteins in filovirus-infected cells, the cells were fixed in 4% paraformaldehyde as described above, washed with 50 mM NH4Cl in PBS and permeabilized with 0.5% Triton X-100 in PBS. After incubation with primary antibodies (rabbit anti-STAT1 or rabbit anti-STAT2 (Santa Cruz; dilution 1:100) along with filovirus-specific antibodies), the specimens were washed with PBS and incubated with fluorescence-labeled secondary antibodies.

To analyze the intracellular localization of endogenous STAT2 in cells expressing individual viral proteins, Huh-7 cells were transfected with 2 μg of plasmid DNA encoding MARV or ZEBOV VP40, VP35, or VP42 using FuGene 6 (Roche) according to the manufacturer’s instructions. VP24 proteins and ZEBOV VP35 were tagged with an HA epitope. As a control cells were transfected with 2 μg pCR3 Flag-tagged rabies virus P. Immunofluorescence analysis was performed by using antibodies directed against STAT2, MARV VP35 (mouse; 1:100), MARV VP40 (mouse; 1:100), ZEBOV VP40 (mouse; 1:100), Flag- (Sigma; dilution 1:700) or HA-tags (Sigma; dilution 1:1000).

VLP budding assay

293T cells were transfected with 2 μg of expression plasmid. At 48 hours post-transfection, cell culture supernatants were clarified by centrifugation at 200 x g for 5 min and pelleted through a 20% sucrose cushion in NTE buffer [100 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA [pH 8.0]] at 160,000 x g for 2 hours at 4°C. Supernatants were aspirated and the pellets containing the
virus-like particles (VLPs) were resuspended in NTE buffer. Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (RIPA) [50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 1% NP-40] supplemented with protease inhibitor cocktail (Roche). VLPs and lysates were analyzed by SDS-PAGE and visualized by western blotting, as described [80].

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Author Contributions

Conceived and designed the experiments: CV OM JO VK. Analyzed the data: MNG MS JO OM VK CB EM. Contributed reagents/materials/analysis tools: SMB. Wrote the paper: CV MNG MS JO CB EM.

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