Virulent poxviruses inhibit DNA sensing by preventing STING activation

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ABSTRACT

Cytosolic recognition of DNA has emerged as a critical cellular mechanism of host immune activation upon pathogen invasion. The central cytosolic DNA sensor cGAS activates STING, which is phosphorylated, dimerises and translocates from the ER to a perinuclear region to mediate IRF-3 activation. Poxviruses are dsDNA viruses replicating in the cytosol and hence likely to trigger cytosolic DNA sensing. Here we investigated the activation of innate immune signalling by 4 different strains of the prototypic poxvirus vaccinia virus (VACV) in a cell line proficient in DNA sensing. Infection with the attenuated VACV strain MVA activated IRF-3 via cGAS and STING, and accordingly STING dimerised and was phosphorylated during MVA infection. Conversely, VACV strains Copenhagen and Western Reserve inhibited STING dimerisation and phosphorylation during infection and in response to transfected DNA and cGAMP, thus efficiently suppressing DNA sensing and IRF-3 activation. A VACV deletion mutant lacking protein C16, thought to be the only viral DNA sensing inhibitor acting upstream of STING, retained the ability to block STING activation. Similar inhibition of DNA-induced STING activation was also observed for cowpox and ectromelia viruses. Our data demonstrate that virulent poxviruses possess mechanisms for targeting DNA sensing at the level of the cGAS-STING axis and that these mechanisms do not operate in replication-defective strains such as MVA. These findings shed light on the role of cellular DNA sensing in poxvirus-host interactions and will open new avenues to determine its impact on VACV immunogenicity and virulence.

IMPORTANCE

Poxviruses are dsDNA viruses infecting a wide range of vertebrates and include the causative agent of smallpox (variola virus) and its vaccine vaccinia virus (VACV). Despite smallpox eradication VACV remains of interest as a therapeutic. Attenuated strains are popular vaccine candidates, whereas replication-competent strains are emerging as efficient oncolytics in
virotherapy. The successful therapeutic use of VACV depends on a detailed understanding of its ability to modulate host innate immune responses. DNA sensing is a critical cellular mechanism for pathogen detection and activation of innate immunity that is centrally coordinated by the ER-resident protein STING. Here STING is shown to mediate immune activation in response to MVA, but not to virulent VACV strains or other virulent poxviruses, which prevent STING activation and DNA sensing during infection and after DNA transfection. These results provide new insights into poxvirus immune evasion and have implications in the rational design of VACV-based therapeutics.
INTRODUCTION

Cells sense the presence of invading pathogens by the use of pattern-recognition receptors (PRRs), a set of germ-line encoded molecules recognising pathogen-associated molecular patterns (PAMPs). Nucleic acids derived from viral infection are potent PAMPs that can be recognised in the cell cytosol and the endolysosomal compartment by dedicated PRRs.

Recognition of RNA or DNA leads to the secretion of type I interferon (IFN) and other inflammatory cytokines and the expression of IFN-stimulated genes (ISGs), which restrict viral replication.

Cytosolic dsRNA is sensed by retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated gene (MDA)-5. Both RIG-I and MDA-5 associate with the IFN-β promoter stimulator (IPS)-1 (also known as MAVS), a protein residing in the mitochondrion that mediates the activation of IFN responsive factors (IRFs) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (1). Cytosolic dsDNA can be sensed by multiple PRRs, the importance of which is largely dependent on the cell type (1). A critical sensor is the cyclic GMP-AMP synthase (cGAS), a nucleotidyltransferase that generates cyclic GMP-AMP (cGAMP) upon binding to dsDNA (2-4).

cGAMP acts as a secondary messenger and docks on stimulator of interferon genes (STING) inducing conformational changes and STING self-association. Upon cGAMP binding STING is phosphorylated and activated and acts as a scaffold for the recruitment of TANK-binding kinase (TBK)-1 and the downstream activation of IRFs and NF-κB (5-8). Besides cGAS, other molecules have been proposed to recognise cytosolic DNA and contribute to STING-dependent IFN responses, including DAI (9), IFI16 (10), the DExD/H-box helicases DHX9, DDX36 (11) and DDX41 (12); and the DNA-damage proteins Ku70/80 (13), DNA-PK (14) and Mre11 (15). Whether and how these molecules impact the cGAS-cGAMP-STING axis and whether they show pathogen and/or cell type specificity are important questions in the field (16, 17).
Poxviruses are a highly successful family of viruses infecting a broad range of species. Besides vaccinia virus (VACV), the prototypic member of the family, and the virus that was used to eradicate smallpox, there are other poxviruses that can cause disease in humans (e.g. the monkeypox virus or the cowpox virus [CPXV]), or in animals (e.g. the rabbit myxoma virus or the mouse ectromelia virus [ECTV]). In addition to virulent strains, non-virulent strains also exist and are popular candidates as recombinant vaccine vectors. Modified vaccinia Ankara (MVA) is an attenuated VACV strain generated through more than 500 serial passages in chicken cells. Through that serial passaging MVA lost its ability to replicate in human cells due to severe deletions and truncations affecting expression of multiple genes compared to the reference strains Copenhagen (COP) or Western Reserve (WR) (18, 19). Accordingly, and in contrast to COP or WR, which retain full inhibitory capacity, MVA has been shown to trigger innate immune activation in multiple experimental settings.

Poxviruses contain large dsDNA genomes of about 200 kbp with a coding capacity for more than 200 proteins. Poxviruses are unique in being the only dsDNA viruses to replicate exclusively in the cell cytoplasm. Replication in this compartment, however, makes this family of viruses particularly susceptible to detection by cytosolic DNA sensors. The success of poxviruses therefore implies the evolution of countermeasures to either avoid recognition or to dampen DNA sensing-induced innate immune activation. An example of such countermeasures is protein C16 which targets DNA-PK and inhibits IRF-3 activation in response to DNA (20). C16 contributes to virulence and is conserved in most VACV strains and poxviruses including variola virus (VARV), but is non-functional in MVA (20, 21). Besides C16, VACV encodes a vast array of immunomodulatory proteins able to inhibit the activation of the IRFs and NF-κB transcription factors (22). Some of these proteins exert their inhibitory action downstream of STING at the level of TBK-1 (23, 24) or IRF-3 (25) and can potentially block STING-mediated induction of type I IFN. However, no VACV
proteins have been reported to specifically target the cGAS-cGAMP-STING axis. To seek evidence for such inhibitors, we investigated the differential capacity of 4 VACV strains to inhibit innate immunity and STING activation in a human monocytic cell line in response to DNA sensing. We demonstrate that virulent VACV strains, as well as CPXV and ECTV, but not the non-virulent strain MVA, encode factors preventing STING phosphorylation and dimerisation during viral infection and upon transfection with exogenous DNA. These findings uncover a novel immune evasion strategy in poxviruses and highlight the importance of DNA sensing in the innate anti-viral defence.

RESULTS

PMA-differentiated THP-1 cells activate IRF-3 in response to MVA, but not COP or WR, infection. To assess the capacity of VACV strains to modulate cellular innate immunity, we sought a cell line that (i) responds to PAMPs; (ii) provides consistent and high-throughput quantitative measurements; and (iii) is amenable to genetic manipulation. THP-1 monocytes expressing Gaussia luciferase (GLuc) under control of the promoter of the IRF-3-dependent gene IFIT-1 (26) provided those characteristics. We differentiated these cells with phorbol 12-myristate 13-acetate (PMA) for 48 h and infected them with 3 different VACV strains at several plaque forming units (PFU) per cell. We monitored GLuc activity over a period of 24 h and plotted it as a fold increase over mock-infected cells. MVA infection triggered IFIT-1-driven GLuc activity and this was quantitated 24 h post-infection (p.i.) at approximately 25-, 20-, and 15-fold increase after infection with 2, 1 and 0.5 PFU/cell, respectively (Fig. 1A). Infection with a higher PFU/cell did not show higher levels of activation (data not shown) possibly due to MVA-induced apoptosis (27, 28).

Conversely, infection with VACV strains COP and WR did not induce GLuc activity. To confirm that these differences were not caused by variations in virus titer, the same sucrose purified stocks were used to infect permissive BHK-21 cells with 5 and 2 PFU/cell. Twelve h p.i. the cells were lysed and lysates were subjected to SDS-PAGE. Immunoblotting against the late viral protein D8
confirmed that infection levels were similar across the different strains (Fig. 1B). We then repeated the infection of THP-1 cells with 2 PFU/cell and measured the induction of CXCL10 and IFN-β mRNA by quantitative PCR 24 h.p.i. MVA infection triggered CXCL10 expression, whereas COP and WR infections did not (Fig. 1C). Production of CXCL10 was subsequently confirmed by ELISA (Fig. 1D). Similarly, we detected a significant increase in IFN-β mRNA expression in response to MVA, but not COP or WR, infection (Fig. 1E). The presence of active IFN-β in the MVA-infected medium was confirmed in a bioassay on HEK293T cells transfected with a reporter expressing luciferase under the control of the IFN-stimulated response element (ISRE). Supernatants from MVA-infected THP-1 cells induced a statistically significant increase in ISRE activity in the HEK293T cells in a dose-dependent manner (Fig. 1F). Form these data we concluded that MVA induces a robust innate immune response in THP-1-IFIT-1-GLuc cells that is not observed with COP or WR, and that measurements of GLuc activity correlate with the upregulation of antiviral cytokines including type I IFN.

**IRF-3 activation in response to MVA infection requires the cGAS-STING axis.** VACV is a dsDNA virus that replicates in the cell cytoplasm and therefore has the potential to be detected by cytosolic DNA sensing mechanisms, in which STING has a pivotal role. To determine whether STING had an impact on the IRF-3 response induced by VACV in THP-1 cells, we transduced THP-1-IFIT-1-GLuc cells with a lentivirus expressing shRNA against cellular STING, or a control shRNA, and selected them with puromycin. Control and shSTING cells were PMA-differentiated and STING levels assessed by immunoblotting, shSTING cells showed reduction in STING expression compared to control cells (Fig. 2A). Furthermore, cells were exposed to cytosolic DNA (herring testes [HT]-DNA transfection) or RNA (Sendai virus [SeV] infection) and IRF-3 responses were determined by luciferase activity. shSTING cells had a significantly impaired response to HT-DNA transfection compared to control cells, but both cell lines responded equally to SeV infection.
which activates IRF-3 via RNA sensors (Fig. 2B). We next measured the response to VACV infection in the STING-depleted cells. Differentiated cells were challenged with MVA at various PFU/cell and luciferase activity was measured 24 h p.i. MVA infection triggered IFIT-1-driven GLuc expression in a dose-dependent manner, and this was impaired in shSTING cells in a statistically significant manner (Fig. 2C).

To further confirm that MVA-induced activation of IRF-3 in THP-1 cells derived from DNA sensing, we generated a THP-1 cell line expressing shRNA against cGAS, the main cellular DNA sensor. Immunoblotting for cGAS confirmed cGAS depletion in shcGAS cells compared to control cells (Fig. 2D). Accordingly, shcGAS cells failed to induce IFIT-1-GLuc expression in response to HT-DNA transfection whilst their response to the RNA sensing activator SeV was unaffected (Fig. 2E). Similar to the results obtained in cells depleted for STING, MVA infection triggered a significantly reduced response in cells depleted for cGAS (Fig. 2F). Together these results demonstrate that cGAS and STING contribute to IRF-3 activation in THP-1 cells infected with MVA.

**COP and WR suppress IRF-3 activation induced by exogenous DNA.** The absence of IRF-3 activation upon COP or WR infection could be explained by (i) an ability of these viruses to mask their dsDNA genome in a manner that is lost in MVA, or (ii) the production of viral factors that prevent IRF-3 activation. We addressed the presence of viral factors by determining the capacity of all 3 VACV strains to inhibit IRF-3 activation mediated by exogenous DNA. First, PMA-differentiated THP-1 cells were infected with 2 PFU/cell of MVA, COP and WR for 6 h and subsequently transfected with HT-DNA. After 16 h HT-DNA transfection induced a ~30-fold increase in IFIT-1-driven GLuc activity in mock-infected and MVA-infected cells, and this was completely suppressed in COP-infected and WR-infected cells (Fig. 3A). We then repeated the infection and measured CXCL10 and IFN-β mRNA expression 6 h after HT-DNA transfection. DNA
challenge triggered expression of CXCL10 (Fig. 3B) and IFN-β (Fig. 3C) in mock-infected cells and this was similar to that observed in MVA-infected cells. COP and WR infections inhibited the DNA-induced expression of both cytokines in a statistically significant manner. Therefore, COP and WR efficiently abolish IRF-3 activation in response to exogenous DNA in THP-1 cells, most likely by the expression of viral factors that are lost or defective in MVA.

**COP and WR inhibit STING phosphorylation in response to DNA.** The ability of VACV virulent strains to block DNA-induced IRF-3 signalling could be ascribed to a cumulative effect of viral inhibitors acting on the IRF-3 pathway, but also to the presence of specific inhibitors acting at the level of cGAS/STING. To address the latter we examined DNA-induced STING activation in VACV-infected cells. A hallmark of STING activation is the phosphorylation of Ser^{366}, an event associated with IRF-3 recruitment and activation (6). We infected PMA-differentiated THP-1 cells with COP or WR for 6 h and subsequently transfected the cells with HT-DNA. Whole-cell lysates were subjected to immunoblotting against p-STING Ser^{366} at 2, 4 and 6 h after DNA transfection. In mock-infected cells, p-STING was detected at 2 h and was prominent at 4 and 6 h post-transfection concomitant with p-IRF-3 induction (Fig. 4A). In infected cells, however, p-STING levels were significantly reduced, particularly after WR infection, and this correlated with a reduction in p-IRF-3.

Immunoblotting against viral protein D8 revealed that the infection levels between COP and WR were similar. Phosphorylated and total STING levels from 3 independent experiments using WR were quantitated and the reduction of p-STING upon DNA challenge was shown to be statistically significant (Fig. 4B).

**COP and WR inhibit STING dimerisation.** To further assess whether WR and COP suppress STING activation, we examined the ability of these viruses to block STING dimerisation in response to DNA (6, 7, 29). Cells were infected with MVA, COP or WR at 2 PFU/cell and
subsequently transfected with HT-DNA for a further 4 h. Cells were lysed and treated to preserve endogenous STING dimers and these were assessed by immunoblotting. In non-stimulated, mock-infected cells a band at ~80 kDa corresponding to the expected size of a STING dimer was detected and became more intense upon DNA sensing stimulation, consistent with the formation of STING dimers in response to exogenous DNA (Fig. 5A). DNA-induced STING dimerisation in MVA-infected cells was indistinguishable from that in mock-infected cells, highlighting the inability of MVA to block STING activation. This notion was also supported by measuring p-STING levels. Conversely, cells infected with COP or WR showed a reduction in DNA-induced STING dimerisation. In agreement with previous observations, p-STING levels were also efficiently suppressed by COP and WR. Levels of viral D8 confirmed similar infectivity between COP and WR. D8 could not be detected in MVA-infected cell lysates due to the fact that MVA does not express late proteins in THP-1 cells. We then repeated this experiment and quantitated the dimeric and monomeric STING bands upon WR infection and DNA stimulation. The results confirmed that WR infection inhibited STING dimerisation in a statistically significant manner (Fig. 5B). Finally, we assessed STING dimerisation and stability upon WR infection at multiple PFU/cell. Infection with as little as 1 PFU/cell was sufficient to prevent DNA-induced STING dimerisation (Fig. 5C). Infection with a higher PFU/cell did not affect the levels of monomeric STING, which remained largely constant and close to those seen in unstimulated cells. Similar data were obtained with COP (Fig. 5D). This suggests that VACV does not affect STING stability. Taken together these data demonstrate for the first time that VACV prevents STING phosphorylation and dimerisation in response to DNA.

**VACV deletion mutant vv811 inhibits STING activation.** VACV protein C16 is expressed by COP and WR and targets DNA-PK (20), a cytosolic DNA sensor recognising VACV and acting via STING (14, 30). To determine whether C16 was responsible for the observed STING inhibition, we
took advantage of vv811, a VACV deletion mutant deriving from COP that lacks 55 open reading frames (ORFs) including C16L (31, 32). Contrary to COP, infection with vv811 triggered ~7 fold increase in IFIT-1-driven GLuc activity (Fig. 6A), suggesting that the absence of immunomodulatory genes limits the ability of vv811 to block IRF-3 responses in differentiated THP-1 cells. vv811 infection reduced the levels of IRF-3 activation observed after DNA transfection as compared to mock-infected cells and this was statistically significant (Fig. 6A). However, vv811 did not reduce IRF-3 activation as effectively as COP, but rather to the levels induced by vv811 infection in the absence of DNA stimulation, suggesting that this baseline activation on infection may be induced by a response that is unrelated to DNA sensing. vv811 inhibition of DNA-induced IRF-3 activation was also observed on measurement of CXCL10 (Fig. 6B) and IFN-β (Fig. 6C) mRNA expression. We then assessed the kinetics of activation of p-STING after HT-DNA transfection in cells infected with vv811 (Fig. 6D). As shown previously, mock-infected cells showed substantial levels of p-STING at 4 and 6 h post-stimulation and these correlated with p-IRF-3 levels. The kinetics of DNA-induced p-STING formation in MVA-infected cells was indistinguishable from that observed in mock-infected cells, implying that no inhibitors of DNA sensing are expressed in MVA. Indeed low levels of p-STING could be detected after MVA infection in the absence of exogenous DNA transfection, indicating that MVA infection is sufficient to trigger STING activation. In contrast, vv811 infection efficiently suppressed both p-STING and p-IRF-3. As expected, viral D8 could not be detected in MVA-infected cells due to its late expression, but was detected in vv811-infected cells demonstrating that this virus replicates in THP-1 cells despite its multiple deletions. Thus, it appears that VACV expresses mechanisms, other than C16, to prevent STING activation in response to DNA sensing.

VACV inhibits STING activation in response to cGAMP. To gain further insight into VACV inhibition of STING-dependent DNA sensing signalling, we assessed the inhibitory capacity of the
different VACV strains in response to cGAMP. Exposure to cGAMP triggered ~6-fold increase in IRF-3-driven reporter activity in PMA-differentiated THP-1 cells (Fig. 7A). This activation was significantly suppressed in cells that had been previously been infected with vv811, COP or WR. Infection with MVA triggered reporter activity on its own and this activity was even enhanced after exposure to cGAMP. To further demonstrate that cGAMP activated STING and this was suppressed by VACV inhibitory strains, we assessed STING dimerisation in response to cGAMP. Exposure to cGAMP induced the formation of STING dimers in mock-infected cells, but these were inhibited in cells infected with WR and even with the deletion mutant vv811 (Fig. 7B). Therefore, irrespective of the potential ability to interfere with cGAS and/or its enzymatic activity, VACV efficiently antagonises the action of STING.

Cowpox and Ectromelia virus prevent STING activation. To determine whether inhibition of STING activation is unique to VACV or occurs after infection with other poxviruses, we studied CPXV and ECTV. Cells were infected with 2 PFU/cell of CPXV reference strain Brighton Red and ECTV reference strain Moscow as well as WR as a control for 6 h, and subsequently stimulated by DNA transfection for a further 4 h. Levels of STING dimerisation were assessed by immunoblotting and viral infection was confirmed by detecting D8 (Fig. 7). As expected, uninfected cells stimulated with DNA showed elevated levels of STING dimers, and these were absent in WR-infected cells. Infection with CPXV and ECTV strains also inhibited STING dimerisation to the same extent as WR, indicating that inhibition of STING activation is conserved amongst multiple orthopoxviruses.

DISCUSSION

STING has emerged as a pivotal molecule in the integration of signals deriving from various cytosolic DNA sensors and in particular cGAS. Upon binding cGAMP STING dimerises, translocates to perinuclear structures and is phosphorylated (5-8). This promotes the
phosphorylation and activation of IRF-3 and the subsequent production of type I IFN and inflammatory cytokines. Whether and how VACV has evolved mechanisms counteracting the cGAS-STING axis is currently unknown. Here we provide evidence demonstrating that VACV inhibits the phosphorylation and dimerisation of STING in response to DNA and cGAMP (Fig. 4, 5 and 7). These observations correlated with the inhibition of IRF-3 activation and of the expression of IFN-β and CXCL10 cytokines (Fig. 1 and 3). Inhibition of STING activation occurred after infection with strains COP and WR, but not MVA. MVA is an attenuated VACV strain unable to replicate and express late genes in most mammalian cell types including human cells. For this reason it is typically titrated on chicken cells (i.e. chicken embryo fibroblasts [CEF]) as opposed to virulent VACV strains which can be titrated on conventional mammalian cell lines (e.g. BS-C-1). Because of this discrepancy we infected permissive BHK-21 cells (in which MVA is fully replicative and hence the late viral protein D8 is expressed) in parallel to differentiated THP-1 cells and observed similar levels of infectivity across the 3 VACV strains (Fig. 1). In PMA-differentiated THP-1 cells MVA infection was sufficient to trigger IRF-3 activation (Fig. 1) and this activation required cGAS and STING (Fig. 2). This data is in line with the reported role of cGAS upon MVA infection in murine dendritic cells (33). MVA is a VACV strain sharing the same dsDNA PAMP as COP or WR but containing up to 6 major genomic deletions (18). A likely explanation for the inability of MVA to block STING activation is that MVA does not express the viral gene(s) responsible for STING inhibition, which are present in COP and WR. However, the possibility existed that the COP and WR genomes were immunologically weaker PAMPS than that of MVA, or that COP and WR were able to mask their genomes more efficiently. Although these possibilities cannot formally be ruled out, the observation that COP and WR blocked STING activation in response to transfected DNA or cGAMP exposure (Fig. 4 and 7) argues that virulent VACV strains express one or multiple factors that are capable of acting in trans to block STING activation induced by the viral genome as well as exogenous DNA. These factors may not operate in MVA, but they (or functionally
equivalent products) must be present in CPXV and ECTV, and presumably in other virulent
poxviruses, because both ECTV and CPXV inhibited STING activation to the same extent as
VACV (Fig. 8).

There are 29 genes absent in MVA due to large genomic deletions and truncations. In addition,
MVA contains non-functional copies of genes due to point mutations and small truncations
acquired during serial passage in chicken cells and does not express late genes due its inability to
complete the viral cycle in most mammalian cells (19, 34, 35). Therefore a large number of VACV
proteins could account for the inhibition of STING activation reported here. An obvious candidate is
protein C16, the viral inhibitor of DNA-PK, which is absent in MVA, but conserved across VACV
replicative strains as well as in CPXV and ECTV (21). Hence we assessed DNA-induced STING
activation during vv811 infection, a VACV deletion mutant that contains 2 large deletions in the
genome terminal regions and does not encode C16 (31). vv811 infection was sufficient to trigger
intermediate levels of IRF-3 activation, consistent with the loss of multiple immune modulators
encoded by the missing genome fragments. Despite this reduced inhibitory capacity, vv811 was as
effective as fully virulent VACV strains in preventing DNA-induced STING activation (Fig. 6). This
indicates that neither C16 nor any of the other 54 ORFs missing in vv811 is required for the
observed STING inhibition, and that additional mechanisms must exist and cooperate with C16 to
effectively block DNA sensing in multiple contexts. Identification of the viral mechanism(s) may thus
require a combination of genetic and biochemical approaches and will be the subject of future
studies.

Whatever the exact mechanism is, it will add to the inhibitory capacity provided by other VACV
factors targeting the IRF-3 signaling cascade downstream of cGAS/STING such as proteins C6
and K7, which act at the level of TBK-1 (23, 24), or protein N2, which blocks IRF-3 activity in the
nucleus (25). VACV redundancy in targeting cellular functions is common and also occurs to prevent NF-κB activation (22) or cell death (36). In the case of NF-κB, vv811 has been shown to inhibit NF-κB activation to the same extent as its parental strain COP despite its large genome deletions (37), and even a recombinant vv811 engineered to lack all described NF-κB inhibitors retained a similar inhibitory capacity as the original vv811, revealing the existence of additional, yet uncharacterised inhibitors (38-40). This remarkable redundancy is necessary to counteract the complex signaling and cross-talk governing innate immune responses in vivo, and indicates that in cells, the contribution of each individual viral protein to the inhibition of a signalling cascade during infection can only be assessed by studying very specific events (i.e. STING phosphorylation and STING dimerisation in this case).

Multiple human viruses target STING function. For instance, the human cytomegalovirus protein UL82 binds to STING and prevents its translocation and activation (41); the Kaposi’s sarcoma-associated herpesvirus (KSHV) protein vIRF1 prevents STING association with TBK-1 (42); the human papillomavirus and adenovirus suppress IFN-β production by targeting STING with their proteins E7 and E1A, respectively (43); whereas Dengue virus does so by cleaving STING via protein NS2B/3 (44, 45). Cleavage is unlikely to underlie the VACV mechanism since our data did not indicate a reduction in STING stability during infection. Other human viruses target cGAS, for example the KSHV proteins ORF52 and LANA (46, 47). At present our data demonstrate that VACV targets STING, but does not exclude that VACV may have evolved complementary mechanisms to interfere with cGAS/STING signalling such as masking the viral DNA with DNA-binding proteins, targeting cGAS or enzymatically degrading cGAMP. The wide array of mechanisms that viruses employ to inhibit the cGAS-STING axis highlights the crucial role of this pathway in innate antiviral defence. In addition, the cGAS-STING pathway has a critical role in inflammatory diseases and in the induction of effective anti-cancer adaptive immunity (48-50).
STING activation is recurrently suppressed in a number of cancers and the level of STING signalling has been shown to correlate with the outcome of VACV or herpesvirus-based oncotherapy (51). The activation and regulation of DNA sensing during poxvirus infection provides a unique model to gain a better mechanistic understanding of DNA-mediated activation of immune responses and will inform in the rationale design of VACV-based therapies for vaccination and oncolytic treatment.

MATERIALS AND METHODS

Cells, reagents and viruses. HEK-293T, BS-C-1, RK-13 and BHK-21 cells were grown in Dulbecco Modified Eagle medium (DMEM, Life Technologies) supplemented with 10 % heat-inactivated fetal calf serum (FCS, Seralab), 100 U/mL penicillin and 100 μg/mL Streptomycin (Pen/Strep, Life Technologies). THP-1-IFIT-1-GLuc cells were a gift from Veit Hornung (University of Munchen, Germany). These cells had been modified to express GLuc under the control of the IFIT-1 promoter (26). THP-1-IFIT-1-GLuc cells were grown in Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies) supplemented with 15 % FCS and Pen/Strep. PMA (Santa Cruz Biotechnology) was dissolved in DMSO at 10 mg/mL. HT-DNA (Sigma) was dissolved in water at 2 mg/mL. 2′3′-cGAMP (Invivogen) was dissolved in water at 50 mg/mL. Sendai virus was a gift from Steve Goodbourn (St George’s University of London, United Kingdom). VACV strains MVA, vv811, COP and WR as well as CPXV strain Brighton Red were obtained from Geoffrey L. Smith (University of Cambridge, United Kingdom). ECTV strain Moscow was from Antonio Alcamí (CBMSO, Spain). MVA was grown and titrated in CEF by conventional plaque assay. All other viruses were expanded in RK-13 or BS-C-1 cells and titrated in BS-C-1 cells. All viruses were purified through a 36 % sucrose cushion before use.
**Reporter gene assays.** THP-1-IFIT-1-GLuc cells were seeded in 96-well plates at a density of 5 × 10⁴ cells per well in the presence of PMA (50 ng/mL). 48 h later cells were infected in quadruplicate with the indicated viruses in RPMI 1640 supplemented with 2 % FCS at the indicated PFU per cell and the medium was collected at the indicated time p.i. When DNA stimulation was performed, PMA-differentiated cells were infected with the indicated viruses at 2 PFU/cell for 6 h and then transfected with HT-DNA at 0.5 μg/mL for a further 16 h. Transfections were performed in OptiMEM (Life Technologies) in the presence of transfection reagent TransIT®-LT-1 (Mirus Bio) following the manufacturer’s recommendations. When cGAMP stimulation was performed, cells were infected as above and then exposed to 10 μg/mL of cGAMP in the medium for a further 16 h. Luciferase activity was measured in a Clariostar plate reader (BMG Biotech) in the presence of coelenterazine (NanoLigh Technology) at 2 μg/mL. Data were normalised to mock-infected samples and presented as a fold increase.

**Quantitative PCR.** THP-1-IFIT-1-GLuc cells were seeded in 24-well plates at a density of 2 × 10⁵ cells per well in the presence of PMA (50 ng/mL). 48 h later cells were infected in triplicate with the indicated viruses in RPMI 1640 supplemented with 2 % FCS at the indicated PFU/cell for 16 h, or for 6 h and subsequently transfected with HT-DNA for a further 6 h as above. RNA was extracted using the Total RNA Purification Kit (Norgen Biotech) and transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. cDNA was diluted 1:5 in water and was used as a template for real-time PCR using SYBR® Green PCR master mix (Applied Biosystems) in a LightCycler® 96 (Roche). Expression of each gene was normalised to an internal control (18S) and these values were then normalized to the non-stimulated mock-infected control cells to yield a fold induction. Primers used for CXCL10 detection were from (52). Primers for 18S detection were: Fwd 5’ GTAACCGTTGAACCCCA 3’ and Rev 5’
CCATCCAATCGGTAGTAGG; and for hIFN-β were: Fwd 5’ ACATCCCTGAGGAGATTAAGCA and Rev 5’ GCCAGGAGGTTCTCAACAATAG.

**ELISA.** Cell culture supernatants from virus-infected THP-1 cells grown in 24-well plates were assayed for CXCL10 using Duoset ELISA reagents (R&D Biosystems) according to the manufacturer’s instructions.

**IFN bioassay.** THP-1-IFIT1-GLuc cells were seeded in 96-well plates at a density of $5 \times 10^4$ cells per well in the presence of PMA (50 ng/mL). 48 h later cells were infected in quadruplicate with the indicated viruses in RPMI 1640 supplemented with 2% FCS at the indicated PFU/cell. Medium was collected 24 h after and transferred to 96-well plate containing HEK293T cells previously transfected for 24 h with 70 ng/well of a reporter plasmid expressing Firefly luciferase under the control of ISRE (Promega) and 10 ng/well of a control plasmid expressing Renilla luciferase (RLuc, Promega) using polyethylenimine (PEI, Sigma) at a ratio 1:2 ($\mu$g DNA:$\mu$L PEI). Activation with recombinant hIFN-β at 25 ng/mL was also included as a control. Ten h after medium transfer, or hIFN-β activation, cells were lysed in Passive Lysis Buffer (Promega). FLuc and RLuc activity was measured in a Clariostar plate reader (BMG Biotech) and FLuc/RLuc ratios were calculated for each well. Data were normalised to mock-infected THP-1 samples and presented as fold increase.

**Generation of cell lines depleted for cGAS and STING.** THP-1-IFIT1-GLuc cells were first depleted for SAMHD1 using specific short hairpin sequences expressed from the HIV-1-based shRNA expression vector HIVSiren (53) and selected for hygromycin resistance (Invivogen, 200 $\mu$g/ml), followed by depletion for STING using the above HIVSiren system, or cGAS using the MLV-based shRNA expression vector pSIREN-RetroQ (Clontech), and selected for puromycin resistance (Merck Chemicals Ltd., 1 $\mu$g/ml). Lentiviral particles were produced by transfection of
HEK293T cells with 1.5 μg pHIVSIREN shRNA, 1 μg p8.91 packaging plasmid (54) and 1 μg vesicular stomatitis virus-G glycoprotein expressing plasmid pMDG (GenScript), or 1.5 μg pSIREN-RetroQ shRNA, 1 μg pCMVi (MoMLV Gag-Pol) and 1 μg pMDG using Fugene 6 transfection reagent (Promega) according to the manufacturer’s instructions. Virus supernatants were harvested 48 h and 72 h post-transfection, pooled and used to transduce THP-1-IFIT-1-GLuc cells.

SAMHD1 shRNA sequence: 5′
CGGGCCATCATCTTGGAATCCAAACTCGAGTTTGGATTCCAAGATGATGGCTTTTT 3′; STING shRNA sequence:
5′GCCTGATAACCTGAGTATGTTCAAGAGACATACTCAGGTTATCAGGCTTTTTTACGCGT 3′;
cGAS shRNA sequence: 5′
GGAAGGAAATGGTTTCCAATTCAAGAGATTGGAAACCATTTCCTTCCTTTTTTACGCGT 3′.

SDS-PAGE and immunoblotting. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (Roche) as well as 250 U/mL benzonase (Sigma). Lysates were rotated for 30 min at 4 °C and subsequently denatured for 5 min at 95 °C in the presence of loading buffer. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare) using a Trans-Blot® semi-dry transfer unit (Bio-Rad).

Membranes were blocked in 0.1 % Tween PBS supplemented with 5 % skimmed milk (Sigma) and subjected to immunoblotting with the following primary antibodies at the indicated dilutions:
- phosphorylated STING Ser366 (Cell Signaling Technology, 1:1,000); STING (Cell Signaling Technology, 1:1,000); cGAS (Cell Signaling Technology, 1:1,000); IRF-3 (Abcam, 1:1,000);
- phosphorylated IRF-3 Ser386 (Abcam, 1:1,000); α-tubulin (Upstate Biotech, 1:10,000); and D8 (a gift from David Ulaeto [DSTL, United Kingdom]). Primary antibodies were detected using IRDye-conjugated secondary antibodies in an Odyssey Infrared Imager (LI-COR Biosciences). Images
were analysed using Odyssey software and data were obtained after integration of at least 3 independent experiments.

**STING dimerisation.** Analysis of STING dimerisation was performed as described (29) with minor modifications. THP-1-IFIT-1-GLuc cells were seeded in 24-well plates at a density of $2 \times 10^5$ cells per well in the presence of PMA (50 ng/mL). After 48 h cells were infected with the indicated viruses in RPMI 1640 supplemented with 2 % FCS at the indicated PFU/cell. After 6 h the cells were transfected with 4 μg/mL of HT-DNA for 4 h or exposed to 15 μg/mL of cGAMP for 6 h and lysed as described above with the exception that lysates were mixed with NuPAGE LDS Sample buffer (Bio-Rad) and directly analysed by SDS-PAGE.

**Statistical analysis.** Statistical significance was determined using an unpaired Student’s t-test with Welch’s correction where appropriate using the GraphPad Prism statistical software.

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**Figure legends**

**FIG 1.** MVA, but not COP or WR, activate IRF-3 and IFN production in PMA-differentiated THP-1 cells. (A) PMA-differentiated THP-1-IFIT-1-GLuc cells were infected in quadruplicate with the indicated PFU/cell (in brackets) of MVA (black), COP (red) or WR (blue), and the medium was analysed for luciferase activity at the indicated times post-infection. Data were normalised to mock-infected samples and presented as a fold increase. (B) BHK-21 cells were infected with the indicated PFU/cell of MVA, COP and WR. Cells were lysed in RIPA buffer and whole cell lysates were subjected to SDS-PAGE and immunoblotting for D8 and α-tubulin. (C, E) PMA-differentiated THP-1-IFIT-1-GLuc cells were infected in triplicate with 2 PFU/cell of MVA, COP or WR, and 16 h later mRNA expression levels of CXCL10 (C) and IFN-β (E) were assessed by qPCR. (D) Medium from cells infected as above with MVA, COP and WR were subjected to ELISA against CXCL10. (F) Medium from cells infected with 2, 1 and 0.5 PFU/cell of MVA or uninfected was transferred to HEK293T previously transfected with an ISRE reporter plasmid (ISRE-FLuc) and a renilla luciferase plasmid (RLuc). Plain medium (n.s.) or medium containing recombinant IFN-β (25 ng/mL) were also used as controls. FLuc/RLuc ratios were normalised to the n.s. control and presented as a fold increase. In all assays, data are presented as mean ± SD and show one representative experiment of at least three. *p<0.05, **p<0.01 or ***p<0.001, unpaired Student’s t-test.

**FIG 2.** Activation of IRF-3 in THP-1 cells upon MVA infection requires STING and cGAS. (A, D) Whole cell lysates from THP-1 cells transduced with lentivirus expressing shRNA against STING and cGAS.
(shSTING) or control (shCtrl) (A), or against cGAS (shcGAS) or control (shCtrl) (D), were subjected to immunoblotting against α-tubulin and STING (A) or cGAS (D). (B, E) PMA-differentiated shSTING or shCtrl (B), or shcGAS or shCtrl cells (E), THP-1 cells were transfected with herring testes (HT)-DNA or infected with Sendai virus (SeV). After 16 h GLuc activity in the supernatant was measured and presented as a fold increase over non-stimulated conditions (n.s.). (C, F) PMA-differentiated shSTING or shCtrl (C), or shcGAS or shCtrl (F), THP-1 cells were infected with 2, 1 and 0.5 PFU/cell of MVA. After 24 h GLuc activity was measured and presented as a fold increase over a mock-infected condition. Data in all graphs are presented as mean ± SD and show one representative experiment of at least three, each performed in triplicate. **p<0.01 or ***p<0.001, unpaired Student’s t-test for the indicated comparisons.

**FIG 3.** VACV strains COP and WR inhibit IRF-3 activation in response to exogenous DNA. PMA-differentiated THP-1 cells were infected with 2 PFU/cell of MVA, COP or WR for 6 h and subsequently transfected with HT-DNA for a further 16 h. (A) GLuc activity was measured and presented as a fold increase over a mock-infected condition. (B-C) Cells were infected as in (A) and subsequently transfected with HT-DNA for a further 6 h. CXCL10 and IFN-β mRNA expression levels were assessed by qPCR. Data are presented as mean ± SD and show one representative experiment of at least three, each performed in triplicate. *p<0.05 or ***p<0.001, unpaired Student’s t-test comparing infections with mock.

**FIG 4.** VACV strains COP and WR inhibit STING phosphorylation in response to DNA transfection. (A) PMA-differentiated THP-1 cells were infected with 2 PFU/cell of the indicated viruses for 6 h and subsequently transfected with HT-DNA (4 µg/mL) for the indicated time. Cells were lysed in RIPA buffer and whole cell lysates were subjected to SDS-PAGE and immunoblotting against the indicated proteins. (B) Ratio of phosphorylated/total STING for the indicated conditions integrating
quantitative data from 3 independent experiments. AU, arbitrary units. *p<0.05, unpaired Student’s t-test for the indicated comparison.

**FIG 5.** VACV strains COP and WR inhibit STING dimerisation in response to DNA transfection. (A) PMA-differentiated THP-1 cells were infected with 2 PFU/cell of the indicated viruses for 6 h and subsequently transfected with HT-DNA for a further 4 h. Cells were lysed in RIPA buffer and whole cell lysates were subjected to SDS-PAGE and immunoblotting against the indicated proteins. (B) Ratio of dimeric/monomeric STING for the indicated conditions integrating quantitative data from 3 independent experiments. AU, arbitrary units. *p<0.05, unpaired Student’s t-test for the indicated comparison. (C-D) PMA-differentiated THP-1 cells were infected with the indicated PFU/cell of WR (C) or COP (D) for 6 h and subsequently transfected with HT-DNA. Cell lysates were subjected to SDS-PAGE and immunoblotting as in (A).

**FIG 6.** vv811 inhibits STING activation. (A) PMA-differentiated THP-1 cells were infected with 2 PFU/cell of MVA, vv811 or COP for 6 h and subsequently transfected with HT-DNA for a further 16 h. GLuc activity was measured and presented as a fold increase over mock-infected conditions. (B-C) Cells were infected as in (A) and subsequently transfected with HT-DNA for a further 6 h. CXCL10 and IFN-β mRNA expression levels were assessed by qPCR. (D) PMA-differentiated THP-1 cells were infected with 2 PFU/cell of the indicated viruses for 6 h and subsequently transfected with HT-DNA (4 µg/mL) for a further 4 h. Cells were lysed in RIPA buffer and whole cell lysates were subjected to SDS-PAGE and immunoblotting against the indicated proteins. One representative experiment of at least 3 is shown. Data in all graphs are presented as mean ± SD, each performed in triplicate. *p<0.05, **p<0.01 or ***p<0.001, unpaired Student’s t-test comparing infection to mock.
FIG 7. VACV inhibits STING activation in response to cGAMP. (A) PMA-differentiated THP-1 cells were infected with 2 PFU/cell of the indicated viruses for 6 h and subsequently exposed to cGAMP a further 16 h. GLuc activity was measured and presented as a fold increase over mock-infected conditions. Data are presented as mean ± SD, each performed in triplicate. ***p<0.001, unpaired Student’s t-test comparing cGAMP-stimulated infections to cGAMP-stimulated mock. (B) PMA-differentiated THP-1 cells were infected with 2 PFU/cell of the indicated viruses for 6 h and subsequently exposed to cGAMP (15 µg/mL) for a further 6 h. Cells were lysed in RIPA buffer and whole cell lysates were subjected to SDS-PAGE and immunoblotting against the indicated proteins. One representative experiment of at least 2 is shown.

FIG 8. CPXV strain Brighton Red (BR) and ECTV strain Moscow (MOS) inhibit DNA-induced STING dimerisation. PMA-differentiated THP-1 cells were infected with 2 PFU/cell of the indicated viruses for 6 h and subsequently transfected with HT-DNA for a further 4 h. Cells were lysed in RIPA buffer and whole cell lysates were subjected to SDS-PAGE and immunoblotting against the indicated proteins. One representative experiment of at least 3 is shown.
