Emerging Alphaviruses Are Sensitive to Cellular States Induced by a Novel Small-Molecule Agonist of the STING Pathway

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ABSTRACT The type I interferon (IFN) system represents an essential innate immune response that renders cells resistant to virus growth via the molecular actions of IFN-induced effector proteins. IFN-mediated cellular states inhibit growth of numerous and diverse virus types, including those of known pathogenicity as well as potentially emerging agents. As such, targeted pharmacologic activation of the IFN response may represent a novel therapeutic strategy to prevent infection or spread of clinically impactful viruses. In light of this, we employed a high-throughput screen to identify small molecules capable of permeating the cell and of activating IFN-dependent signaling processes. Here we report the identification and characterization of *N*-(methylcarbamoyl)-2-{[5-(4-methylphenyl)-1,3,4-oxadiazol-2-yl]sulfanyl}-2-phenylacetamide (referred to as C11), a novel compound capable of inducing IFN secretion from human cells. Using reverse genetics-based loss-of-function assays, we show that C11 activates the type I IFN response in a manner that requires the adaptor protein STING but not the alternative adaptors MAVS and TRIF. Importantly, treatment of cells with C11 generated a cellular state that potently blocked replication of multiple emerging alphavirus types, including chikungunya, Ross River, Venezuelan equine encephalitis, Mayaro, and O'nyong-nyong viruses. The antiviral effects of C11 were subsequently abrogated in cells lacking STING or the type I IFN receptor, indicating that they are mediated, at least predominantly, by way of STING-mediated IFN secretion and subsequent autocrine/paracrine signaling. This work also allowed characterization of differential antiviral roles of innate immune signaling adaptors and IFN-mediated responses and identified MAVS as being crucial to cellular resistance to alphavirus infection.

IMPORTANCE Due to the increase in emerging arthropod-borne viruses, such as chikungunya virus, that lack FDA-approved therapeutics and vaccines, it is important to better understand the signaling pathways that lead to clearance of virus. Here we show that C11 treatment makes human cells refractory to replication of a number of these viruses, which supports its value in increasing our understanding of the immune response and viral pathogenesis required to establish host infection. We also show that C11 depends on signaling through STING to produce antiviral type I interferon, which further supports its potential as a therapeutic drug or research tool.

KEYWORDS chikungunya virus, IRF3, interferon, Mayaro virus, O'nyong-nyong virus, Ross River virus, STING, Venezuelan equine encephalitis virus, alphavirus, antiviral agents

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The type I interferon (IFN) system is a rapidly mobilized line of defense against invading microbes that initiates and directs cellular and systemic processes to eliminate pathogenic threats. Type I IFNs are secreted cytokines that include IFN-β and multiple IFN-α subtypes that engage the IFN-α/β receptor (IFNAR) present on nearly all cells (1). Intracellular signaling that ensues involves phosphorylation of signal transducer and activator of transcription 1 and 2 (STAT1/2) via Janus kinases (JAKs) and leads to transcription of myriad IFN-stimulated genes (ISGs). These encode immunologically active proteins, including direct antiviral effectors (2) exhibiting molecular functions that impair multiple cell-based processes essential to replication of diverse viruses, and are ultimately required for clearance of infection (3). Importantly, the type I IFN system in vertebrates represents a mechanism to block the intrahost growth of viruses across broad taxonomic classes and irrespective of their pathogenic potential. IFN-stimulated cellular states are thus refractory to replication by both known and unknown virus types, including those that are potentially zoonotic or emerging. Pharmacologic stimulation of IFN processes is therefore being investigated as an antiviral strategy that may bestow clinically impactful broad-spectrum outcomes, especially during outbreaks of unidentified agents (4).

IFN synthesis is initially triggered following detection of pathogen- or danger-associated molecular patterns (PAMPs and DAMPs, respectively) by germ line-encoded pattern recognition receptor (PRR) proteins. PAMPs and DAMPs are biochemically diverse and include structural components of microbes as well as biosynthetically derived molecules, such as nucleic acids and cyclic dinucleotides (CDNs) (5). PRRs upstream of IFN transcription include transmembrane Toll-like receptors (TLRs) of double-stranded RNA (dsRNA) (TLR3) or bacterial lipopolysaccharide (LPS) (TLR4) as well as cytoplasmic sensors, such as RIG-I-like receptors (RLRs) of dsRNA and cytoplasmic dsDNA receptors (CDRs) (6, 7). PRR engagement leads to induction of phosphorylation- and ubiquitination-driven signaling that activates transcription factors, such as IFN regulatory factor 3 (IRF3) and nuclear factor κB (NF-κB), that are essential to synthesis of mRNAs encoding IFN-β and proinflammatory cytokines/chemokines as well as antimicrobial effectors (8, 9). Importantly, canonical PRR-induced pathways involve specific adaptor proteins that integrate upstream signals from particular receptors and impart degrees of specificity to the resultant transcriptional program (10) in ways that are poorly understood. Key adaptors of IRF3/IFN-terminal pathways include mitochondrial antiviral signaling protein (MAVS; also called IPS-1, VISA, and CARDIF), which is necessary for the dsRNA-dependent PRRs RIG-I and MDA5 (11); TIR domain-containing adaptor inducing IFN-β (TRIF; also called TICAM1), which is utilized by TLR3 and TLR4 (12); and STING (also called MITA, ERIS, and TMEM173), which is both an endoplasmic reticulum-associated PRR for CDNs (13) and an adaptor for CDRs (14). MAVS, TRIF, and STING activate the kinases IKK and TBK1, which activate NF-κB and IRF3, respectively (15). Individual stimuli can engage multiple PRR-adaptor pathways and thus generate responses that are complex and surprisingly unexplored (16, 17). Moreover, synthetic small molecules have been identified that stimulate these processes with great precision and elicit IFN-mediated immunotherapeutic effects, including impairment of virus replication (18–23).

Mosquito-transmitted alphaviruses are members of the *Togaviridae* family that are distributed widely throughout the globe and have historically demonstrated a tendency to spontaneously emerge in susceptible human populations (24, 25). This is best exemplified by chikungunya virus (CHIKV), which was first described in 1953 but reemerged dramatically in 2004, causing massive epidemics on islands of the Indian Ocean and the Indian mainland and eventually spreading to the New World in 2013 (26). Emergent outbreaks in recent history have also been documented for infections with other alphaviruses, including Venezuelan equine encephalitis virus (VEEV) (27), Ross River virus (RRV) (28), Mayaro virus (MAYV) (29), and O'nyong-nyong virus (ONNV) (30). Clinical manifestations of infections with alphaviruses evolutionarily derived from the Old World (CHIKV, RRV, ONNV, and MAYV) primarily include an acute febrile episode followed by polyarthralgia and myalgia that can be severe and long lasting (reviewed in reference 31). Infections with New World alphaviruses, such as VEEV, are also febrile

but can lead to severe and often fatal neurologic disease, including encephalitis (32). Importantly, there currently exist no FDA-approved antiviral drugs or vaccines to prevent alphavirus-associated disease.

Alphaviruses exist as enveloped particles that contain a positive-sense, single-stranded RNA genome encoding four nonstructural and five structural proteins (33). Infection of host cells is associated with a rapid and strong induction of innate signaling pathways, including those leading to activation of IRF3 (34, 35). This occurs through detection of virus-associated dsRNA by cytoplasmic PRRs, such as RIG-I and MDA5, which signal to IRF3 via MAVS (34, 36–38), as well as through protein kinase R (PKR), which inhibits the cellular translational machinery through phosphorylation-based inactivation of eukaryotic initiation factor 2α (34, 39). Despite this, alphaviruses are extremely susceptible to the effects of type I IFNs and replicate poorly in cells in which an IFN-induced state has been elicited (reviewed in references 40 and 41). To counteract these effects, these viruses have evolved mechanisms to globally inhibit synthesis of cellular mRNAs and proteins, especially those stimulated by IFN-dependent signaling (reviewed in reference 42). As such, IFN exhibits its optimal antiviral effect when the cytokine is exposed to target cells prior to viral entry. Importantly, direct susceptibility to IFN-induced cellular states is documented for CHIKV, VEEV, MAYV, ONNV, and RRV. Based on this, we decided to pursue a unique approach to investigate the sensitivity of emerging alphavirus types to innate stimulation via IRF3/IFN-terminal pathways. For this purpose, we explored the phenotypes and host targets of a novel small molecule that activates innate immune responses in human cells. In addition to representing a molecular tool for investigating the impact of innate immune stimulation on alphavirus replication, this may also lead to development of new antiviral drugs or therapeutic strategies for viral control.

RESULTS

C11 is a small molecule that induces type I IFN-dependent luciferase activity in human fibroblast and myeloid cell lines. To identify novel small molecules capable of activating the type I IFN response in human cells, we employed a high-throughput screening assay developed using telomerase-transduced foreskin fibroblasts (THF) that were stably transduced with green fluorescent protein (GFP) and luciferase (LUC) reporter proteins responsive to IFN-induced, ISRE-dependent signaling (18, 43). Examination of approximately 52,000 chemically diverse molecules in duplicate led to identification of *N*-(methylcarbamoyl)-2-{[5-(4-methylphenyl)-1,3,4-oxadiazol-2-yl]sulfanyl}-2-phenylacetamide, which we have termed C11, as the most potent overall hit. We next confirmed the screening results in THF and myeloid-derived MonoMac6 (MM6) cells transduced with the ISRE-LUC reporter (THF-ISRE and MM6-ISRE cells, respectively) and examined whether the LUC signal correlated with the molecule's dose. As shown in Fig. 1A, LUC activity in THF-ISRE cells exceeded the background at 25 μM C11 and increased proportionally to dosage, to a maximum of approximately 60-fold. LUC activity in MM6-ISRE cells exceeded the background at 50 μM C11 and increased to 50-fold above that for mock treatments at 100 μM C11 (Fig. 1B). Importantly, cell viability as determined by ATP release was not significantly affected at any concentration after 24 h (Fig. 1A and B).

Next, we examined the ability of C11 to elicit innate reactivity in nonhuman cells. For this purpose, we utilized murine RAW264.7 monocytic cells stably transduced with an ISRE-LUC reporter cassette as shown in Fig. 1C. While the mouse-specific IFN-inducing molecule DMXAA (5,6-dimethylxanthenone-4-acetic acid) was able to trigger a substantial LUC signal in these cells, no induction was seen with any dose of C11. Since C11 elicited responses in myeloid-derived MM6 cells, we next asked whether another human monocytic cell line reacts similarly to the compound. Surprisingly, phorbol myristate acetate (PMA)-differentiated THP-1 cells stably transduced with an IFN-dependent reporter responded to transfection with 2′3′-cGAMP but not to treatment with C11 (Fig. 1D). These results indicate that the molecule does not activate an innate immune response in murine cells or THP-1 cells but does so in human fibroblasts and MM6 cells. This suggests that the host factors targeted by the molecule that lead to

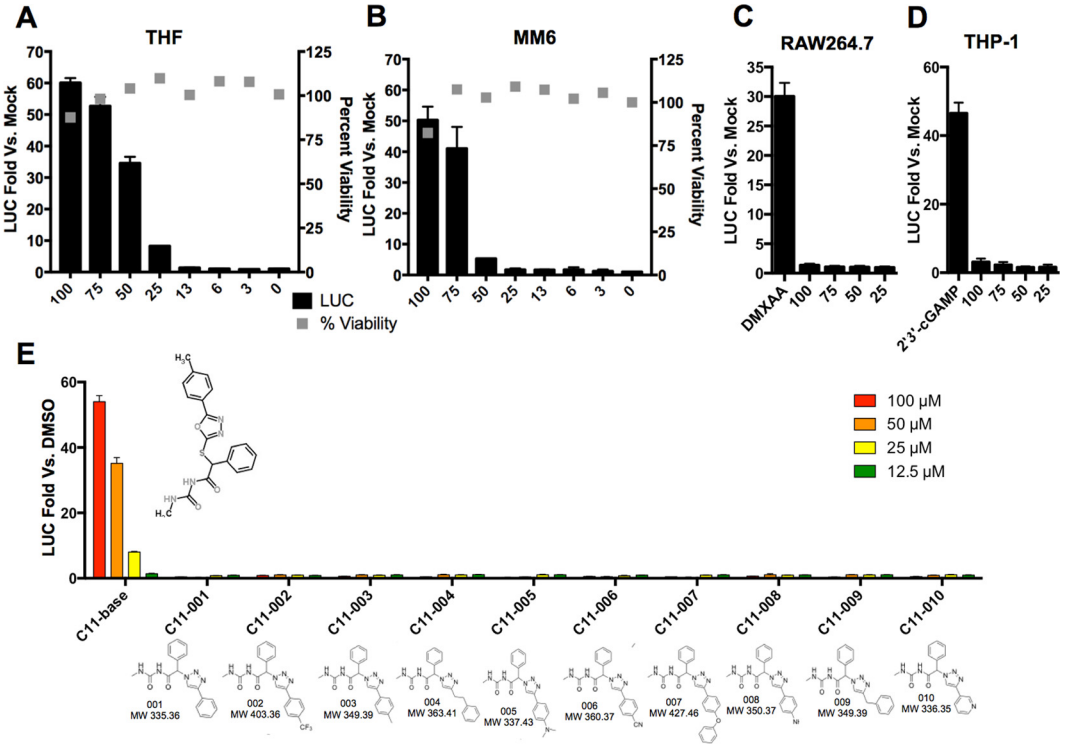

FIG 1 Dose-dependent activation of type I interferon-mediated signaling and cytotoxicity of C11. The top graphs show ISRE-dependent expression of luciferase (LUC) as well as percent cellular viability as determined by CellTiter-Glo assay for THF (A) and MM6 (B) cells and ISRE-dependent expression of LUC in RAW264.7 (C) and THP-1 (D) cells (the *x* axis represents the C11 concentration in micromolar units). Cells were exposed to C11 at the indicated concentrations (micromolar), 10 μg/ml 2′3′-cGAMP, or 50 μM DMXAA for 8 h (LUC assay) or 24 h (CellTiter-Glo assay). Values presented are mean fold changes and standard deviations (SD) relative to the values for cells treated with 1% DMSO (black bars; left *y* axis). Cell viability data are expressed as percentages of the signal detected in DMSO-treated cells (gray squares; right *y* axis). Values displayed are based on three replicates. (E) Chemical structure of *N*-(methylcarbamoyl)-2-{[5-(4-methylphenyl)-1,3,4-oxadiazol-2-yl]sulfanyl}-2-phenylacetamide (C11). The graph shows luminescence from THF-ISRE cells following 8 h of exposure to multiple concentrations of the indicated C11 derivative molecules, with structures shown below the graph. Values presented are average fold changes and SD for duplicates relative to the values for DMSO-treated cells.

IFN-dependent activity either are lacking in nonresponsive cells or exist as variants that are nonreactive to C11 stimulation. These are discussed in more detail below. To obtain knowledge about C11's structure-activity relationship (SAR), such as which moieties may be essential to the compound's innate activity, we next synthesized 10 analogs of the molecule that differed from the original at various positions (Fig. 1E). THF-ISRE cells were exposed to these analogs at 12.5, 25, 50, or 100 μM for 8 h, and LUC activities were measured. Unfortunately, all of the chemical alterations we attempted led to abrogation of innate immune stimulation by the derivatives. These results suggest that either the structure of C11 is chemically optimal for innate immune activation or the modifications made were qualitatively inconsistent with maintaining the compound's innate stimulatory capacity. To address this, additional analogs could be synthesized and tested as described here.

C11 induces transcription and translation of interferon-dependent antiviral genes. Since C11 triggered expression of a heterologous ISRE-dependent reporter protein, we next asked whether it was also able to induce transcription of endogenous host genes, including type I IFN genes and ISGs. As shown in Fig. 2A, treatment of THF cells with C11 led to upregulation of Viperin (RSAD2) and IFIT1 mRNAs to levels similar to those induced by treatment with IFN-β or the IFN-inducing RNA virus Sendai virus (SeV). Moreover, C11 also induced expression of IFN-β itself, a result consistent with observed ISG induction. Interestingly, transfection with 2′3′-cGAMP, the endogenous ligand for the STING protein, also triggered transcription of IFN-β but induced substantially higher levels of IFIT1 and Viperin, suggesting differential activation processes

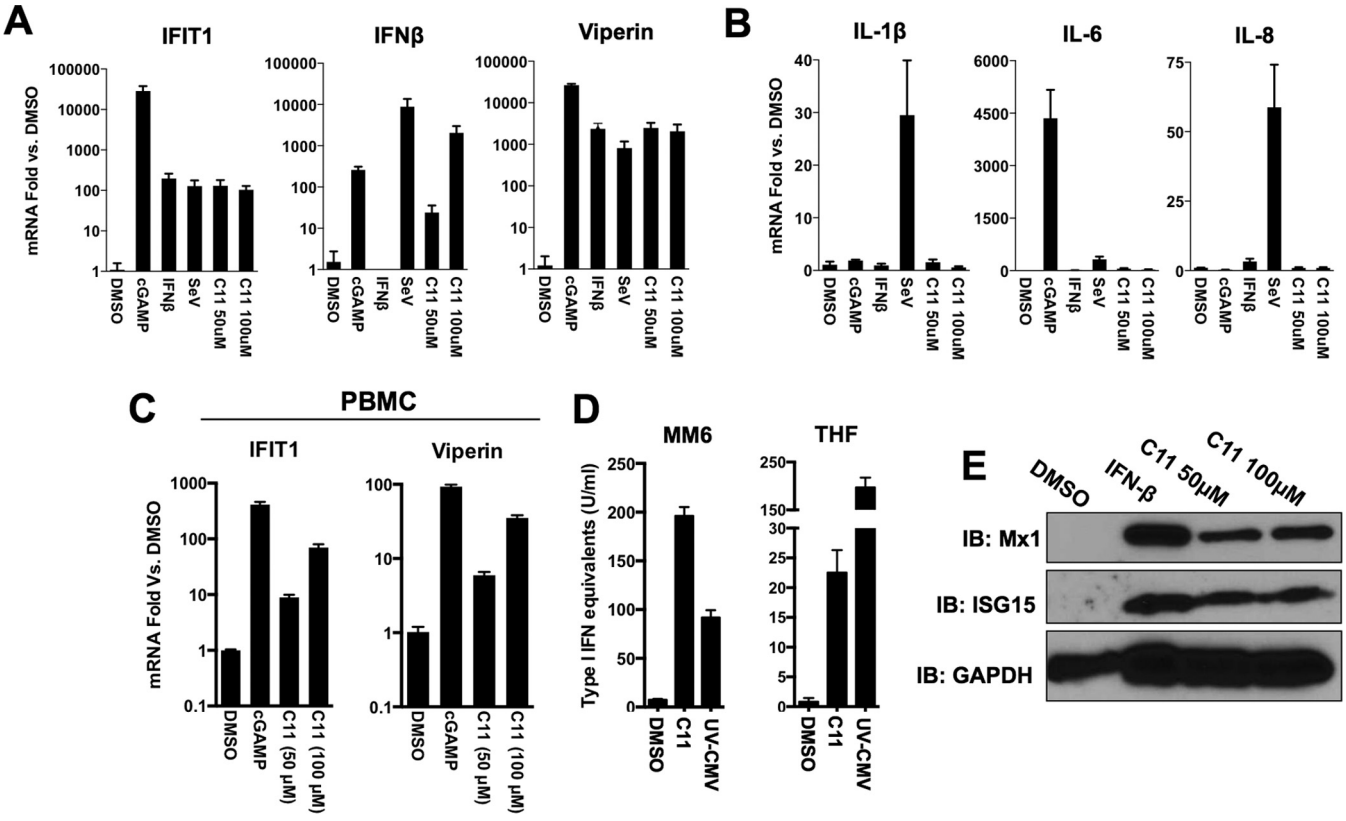

FIG 2 C11 induces transcription and translation of IFN- but not NF-κB-dependent genes. Average fold changes and SD (relative to the values for cells treated with 1% DMSO) for duplicate experiments are shown for IFIT1, Viperin, and IFN-β (A) or IL-1β, IL-6, and IL-8 (B) mRNAs in THF cells following 6 h of exposure to IFN-β (1,000 U/ml), SeV (160 HAU/ml), cGAMP (10 μg/ml), or C11 (50 μM or 100 μM). (C) Average fold changes and SD (relative to the values for cells treated with 1% DMSO) for duplicate experiments for IFIT1 and Viperin mRNAs in human PBMC following 8 h of exposure to 1% DMSO, 10 μg/ml 2′3′-cGAMP, or C11 (50 μM or 100 μM). (D) Secretion of bioactive type I IFN from MM6 and THF cells treated in triplicate overnight with 1% DMSO, C11 (50 μM), or UV-inactivated CMV (MOI = 3). Data are expressed as average levels and SD for type I IFN equivalents. (E) Immunoblots (IB) of whole-cell lysates of THF-ISRE cells following 8 h of exposure to 1% DMSO, IFN-β (1,000 U/ml), or C11 (50 μM or 100 μM), as indicated, showing protein levels of Mx1, ISG15, and GAPDH.

by or cellular sensitivity to these stimuli. Synthesis of IFN-β mRNA often correlates with that of proinflammatory genes due to the involvement of PRR-induced signaling pathways that activate transcription factors required for both, such as IRFs and NF-κB (8, 44). We therefore examined expression of interleukin-1β (IL-1β), IL-6, and IL-8 in these cells in response to C11 treatment. As shown in Fig. 2B, the molecule failed to induce significant levels of mRNAs for these genes, in contrast to SeV, indicating a likely disparity in the proinflammatory signaling pathways triggered by these stimuli. Intriguingly, however, while 2′3′-cGAMP did not induce detectable IL-1β or IL-8 mRNA synthesis, it led to very high levels of IL-6 mRNA, a phenomenon not previously described, to our knowledge. We next asked if C11 would induce the transcription of ISGs in primary human cells by treating human peripheral blood mononuclear cells (PBMC). As shown in Fig. 2C, the compound similarly activated expression of ISGs in these cells, indicating that its stimulatory capacity is evident in cells beyond those with extended life.

Since IFN-dependent proteins are ultimately responsible for conferring antiviral phenotypic effects, we next examined the translation of C11-associated genes. We first confirmed that C11 was able to elicit secretion of bioactive type I IFN in THF and MM6 cells. As shown in Fig. 2D, exposure of THF and MM6 cells to 50 μM C11 resulted in significant levels of functional type I IFN in the culture media, as did the control stimulus, human cytomegalovirus rendered inactive by UV irradiation (UV-CMV). In addition, C11 exposure also led to translation of ISG proteins Mx1 and ISG15 (Fig. 2E), both known to confer potent antiviral activity (45–47). As such, we concluded that C11

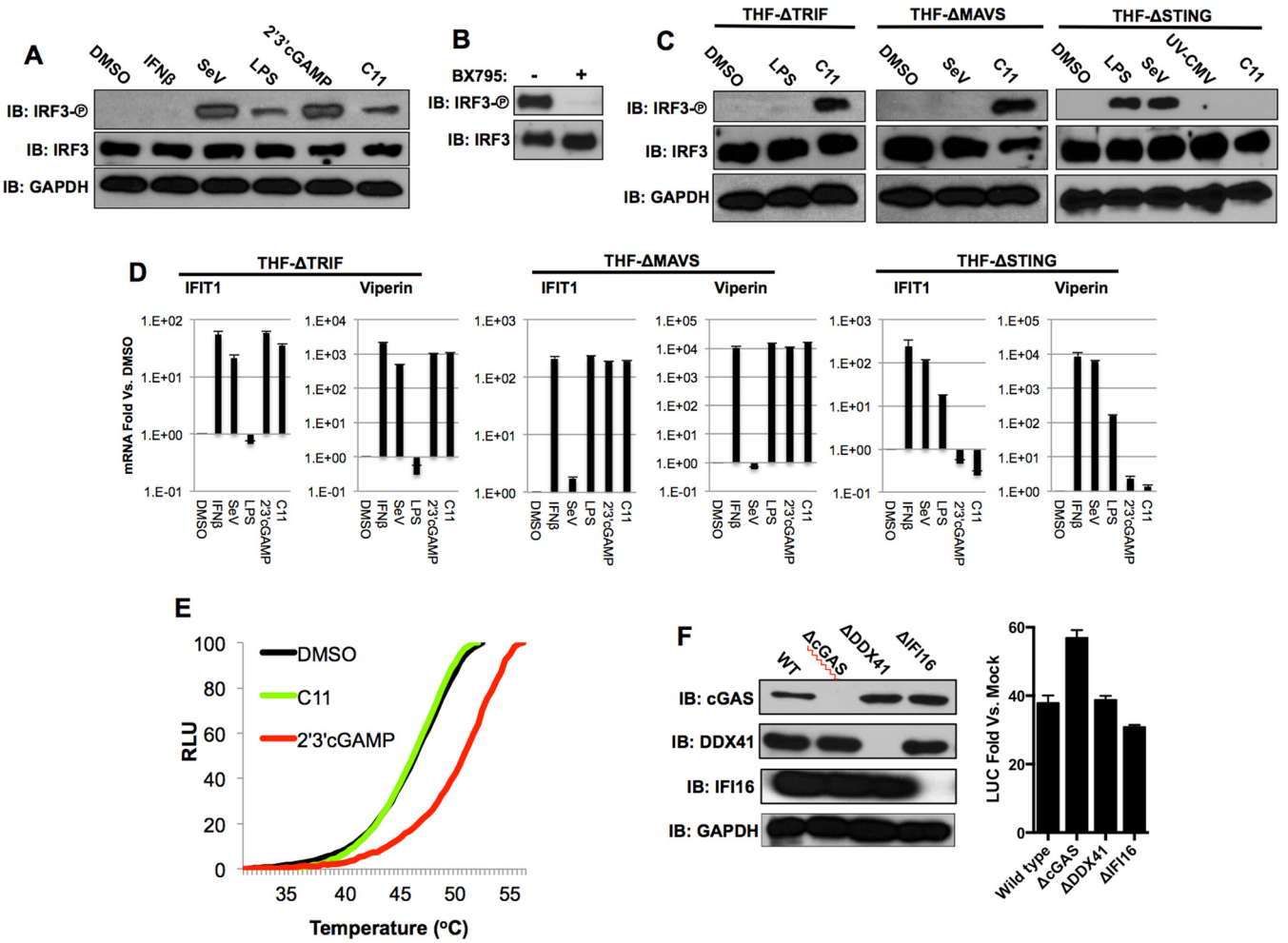

FIG 3 C11 induces IRF3 phosphorylation and ISG transcription in a manner that requires STING but is independent of MAVS, TRIF, or CDRs. (A) Immunoblots of whole-cell lysates of THF-ISRE cells following 4 h of exposure to 1% DMSO, IFN-β (1,000 U/ml), SeV (160 HAU/ml), LPS (10 μg/ml), 2′3′-cGAMP (10 μg/ml), or C11 (75 μM), as indicated, showing the phosphorylation status of IRF3 S386, total IRF3, and GAPDH. (B) Immunoblots of whole-cell lysates showing phosphorylation status of IRF3 S386 and total IRF3 for wild-type THF cells pretreated for 2 h with DMSO or BX795 (TBK1 inhibitor; 10 μM) and then stimulated with C11 (75 μM) for 4 h. (C) Immunoblots of whole-cell lysates of cells lacking TRIF, MAVS, or STING following 4 h of exposure to 1% DMSO, SeV (160 HAU/ml), LPS (10 μg/ml), 2′3′-cGAMP (10 μg/ml), UV-CMV (MOI = 3), or C11 (75 μM), as indicated, showing phosphorylation status of IRF3 S386, total IRF3, and GAPDH. (D) Average fold changes and SD (relative to the values for cells treated with 1% DMSO) for duplicate experiments for IFIT1 and Viperin mRNAs in THF cells lacking TRIF, MAVS, or STING following 8 h of exposure to the indicated treatments, as described above. (E) Melting temperature shifts for human STING-CTD in the presence of DMSO, 100 μM C11, or 200 μM 2′3′-cGAMP. RLU, relative light units. (F) (Left) Immunoblot showing presence or absence of cGAS, DDX41, or IFI16 protein in each THF-ISRE cell line, as well as GAPDH as a loading control. (Right) Luminescence from WT parental THF-ISRE cells as well as that from cells with deletions created using CRISPR/Cas9 technology. Values presented are average fold changes and SD for duplicates relative to the value for DMSO-treated cells.

is able to trigger transcription and translation of type I IFN genes, as well as canonical ISGs, but not those of proinflammatory genes.

C11 induces canonical IRF3 phosphorylation and ISG transcription in a manner that requires STING but not MAVS or TRIF. Transcription of IFN-β requires IRF3 in an activated form, as indicated by the phosphorylation of C-terminal serine residues that occurs via the kinase TBK1 (48). Since C11 induces IFN secretion and ISG transcription, we predicted that it would also trigger phosphorylation of IRF3 via this mechanism, as do other established IFN-inducing stimuli. As shown in Fig. 3A, exposure of THF cells to canonical IRF3-terminal PAMPs, such as SeV (MAVS agonist), LPS (TRIF agonist), and 2′3′-cGAMP (STING agonist), led to phosphorylation of IRF3 S384 by 4 h postinfection. Likewise, C11 triggered a similar response, indicating that the molecule also stimulates an innate signaling reaction that leads to IRF3 activation. Next, to verify that C11 activates the TBK1-IRF3 axis, we pretreated THF cells with BX795, a small-molecule

inhibitor of TBK1 (49), prior to cotreatment with C11. BX795 treatment resulted in nearly complete suppression of IRF3 phosphorylation, indicating that C11 triggers canonical TBK1-dependent phosphorylation of IRF3 (Fig. 3B).

TBK1-mediated IRF3 phosphorylation occurs in response to one or more activated signaling cascades defined by the adaptor proteins MAVS, TRIF, and STING (reviewed in reference 10). To determine the target pathway stimulated by C11 upstream of TBK1, we tested whether C11-mediated IRF3 activation requires any of these adaptors by employing three THF cell lines, each lacking a single adaptor protein, to perform loss-of-function assays (18, 43). As illustrated in Fig. 3C, cells lacking TRIF or MAVS failed to exhibit IRF3 phosphorylation in response to established stimuli of those specific adaptors (LPS and SeV, respectively), therein functionally demonstrating the absence of the proteins. However, IRF3 phosphorylation was observed in both cell types following exposure to C11, suggesting that the molecule activates a pathway(s) that requires neither protein or requires either MAVS or TRIF in a redundant manner. However, while cells lacking STING exhibited IRF3 phosphorylation in response to TRIF- and MAVS-specific stimuli (LPS and SeV), no such reactivity was observed following exposure to UV-inactivated cytomegalovirus (a demonstrated STING agonist [50]) or C11. These observations both verify the absence of STING-mediated signaling in these cells and indicate that C11 requires a STING-inclusive pathway for its phosphorylation of IRF3.

We next examined synthesis of ISG mRNAs (IFIT1 and Viperin) in these cells to validate that their transcription correlates with expected responses based on IRF3 activity. As shown in Fig. 3D, all cell types transcribed IFIT1 and Viperin in response to IFN-β, indicating that the IFN-dependent JAK/STAT pathways were intact in all cases. Moreover, exposure to canonical activators of the adaptors encoded by the cells also led to ISG induction as evidence that those pathways were also functional. As expected, stimuli specific for pathways defined by the deleted proteins failed to induce significantly greater expression of the ISGs than that with dimethyl sulfoxide (DMSO), functionally confirming the absence of each adaptor protein. In agreement with our observations regarding IRF3 phosphorylation responses (Fig. 3C), the STING agonist 2′3′-cGAMP and C11 led to ISG transcription in cells lacking MAVS or TRIF but not STING (Fig. 3D). This result provides additional confirmation that STING, but not MAVS or TRIF, is essential to the innate signaling response induced by the molecule.

We next examined whether evidence exists that C11 may activate STING-dependent processes through direct engagement of the protein. For this purpose, we employed a fluorescence-based thermal shift assay on a purified protein consisting of the STING C-terminal ligand binding domain (LBD) (43). In this procedure, we expect that direct contact between the protein and an interrogated ligand will result in an increase in the protein's thermal stability that is manifested as emission of SYPRO Orange at higher temperatures than those in the absence of the ligand. As shown in Fig. 3E, incubation of purified STING-LBD with 2′3′-cGAMP led to an obvious increase in the temperature at which fluorescence was emitted relative to that with DMSO alone. However, the presence of C11 did not lead to a detectable change in fluorescence emission relative to that induced by DMSO. This observation is inconsistent with C11 directly interacting with STING-LBD as 2′3′-cGAMP does.

Given that C11 is unlikely to act as a direct STING ligand, we next asked whether the molecule may require a STING-dependent PRR for innate immune activation. To address this, we used clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 genome editing to construct three THF-ISRE cell lines that singularly lack cGAS, DDX41, or IFI16, as illustrated in Fig. 3F. Treatment of each of these cells with 75 μM C11 induced LUC expression that resembled that observed in the wild-type (WT) parental cells. Based on this result, we concluded that C11 activates innate immune signaling in a manner that requires STING but not cGAS, DDX41, or IFI16, PRRs that have been shown to signal by way of STING.

C11-mediated IRF3 and ISG activation occurs in cells lacking both MAVS and TRIF. The results presented above indicate that the IRF3/IFN-activating response triggered by C11 requires STING but neither MAVS nor TRIF individually. It is still

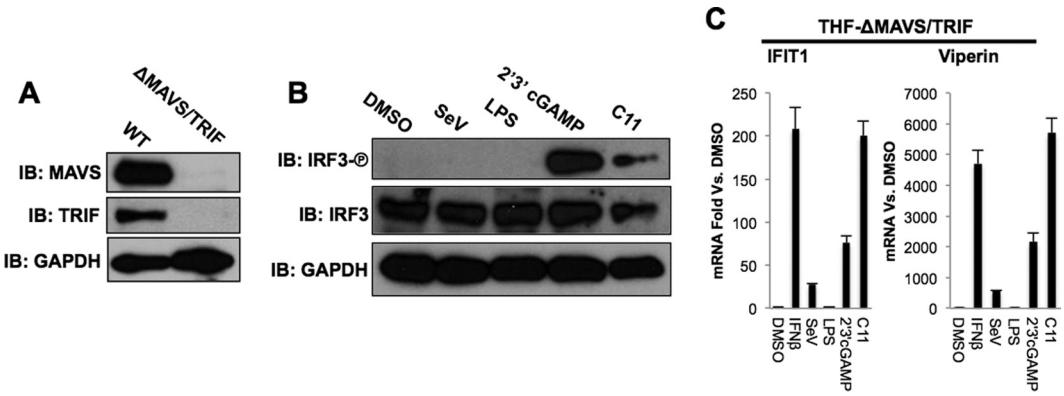

FIG 4 C11 induces IRF3 phosphorylation and ISG transcription in a manner that is independent of MAVS and TRIF. (A) Immunoblots of whole-cell lysates for MAVS, TRIF, and GAPDH for parental THF-ISRE (WT) cells as well as derivative cells in which the MAVS and TRIF proteins were deleted (THF-ISRE-ΔMAVS/TRIF cells) by use of CRISPR/Cas9 technology. (B) Immunoblots of whole-cell lysates of THF-ISRE-ΔMAVS/TRIF cells following 4 h of exposure to 1% DMSO, SeV, LPS, 2′3′-cGAMP, UV-CMV (MOI = 3), or C11 (75 μM), as indicated, showing phosphorylation status of IRF3 S386, total IRF3, and GAPDH. (C) Synthesis of IFIT1 and Viperin mRNAs in THF-ISRE-ΔMAVS/TRIF cells following exposure to 1% DMSO, 1,000 U/ml IFN-β, 160 HAU/ml SeV, 10 μg/ml LPS, 10 μg/ml 2′3′-cGAMP, or 75 μM C11. Values presented are average fold changes and SD for duplicate experiments relative to the values for cells treated with 1% DMSO.

possible, however, that innate activation by the compound requires either MAVS or TRIF as well as STING. Precedents demonstrating essential involvements of both MAVS (reviewed in reference 51) and TRIF (52) in specific STING-mediated reactions do exist. To address whether C11 activates a STING-inclusive signaling response that requires either TRIF or MAVS redundantly, we constructed a THF cell line that lacks both proteins (Fig. 4A). As predicted, these cells did not exhibit IRF3 phosphorylation (Fig. 4B) or ISG transcription (Fig. 4C) in response to treatment with SeV or LPS, yet they did display IRF3 phosphorylation following treatment with 2′3′-cGAMP, indicating that STING-mediated signaling was operational. Importantly, C11 was also able to activate IRF3 phosphorylation (Fig. 4B) and ISG induction (Fig. 4C) in these cells. This observation supports the conclusion that neither TRIF nor MAVS is redundantly essential in combination with STING for the IRF3-terminal pathway triggered by C11.

C11 elicits a cellular state that inhibits replication of multiple emerging alphaviruses. Since exposure of cells to C11 leads to expression of type I IFN and ISGs known as antiviral effectors, we next asked whether the compound can generate a cellular environment antagonistic to virus growth. For this purpose, we chose to examine members of the *Alphavirus* genus, since they exhibit great sensitivity to the effects of IFN (reviewed in references 40 to 42) and are highly clinically relevant based on their propensity for spontaneous emergence. We therefore measured growth of CHIKV, VEEV, MAYV, RRV, and ONNV on THF cells over a range of C11 concentrations. As shown in Fig. 5A, viral titers of each virus decreased significantly with increasing C11 molarity. This resulted in 90% effective concentration (EC_{90}) values of 16.44 μM for CHIKV, 16.7 μM for VEEV, 18.84 μM for ONNV, 25.19 μM for MAYV, and 22.57 μM for RRV. Titers of CHIKV and ONNV dropped over 5 and 4 logs, respectively, to below the limit of detection, with 50 μM C11. Titers of VEEV and MAYV dropped approximately 4 logs but were detectable with 50 μM C11. RRV was the least responsive to C11 in terms of titer decrease but also grew to the lowest titer of all examined viruses in the absence of the compound, a likely effect of its sensitivity to the inherent innate immune capacity of THF cells (addressed below). Based on these results, we concluded that C11 elicits conditions in THF cells that are potently refractory to growth of multiple alphavirus types.

The data in Fig. 5A indicate that C11 prophylactically generates a cellular state that is inhibitory to replication of multiple alphavirus types. We next decided to ask if C11 can impair virus replication on exposure of cells to the compound following infection (therapeutic efficacy). For this purpose, we focused on CHIKV and VEEV because (i) they

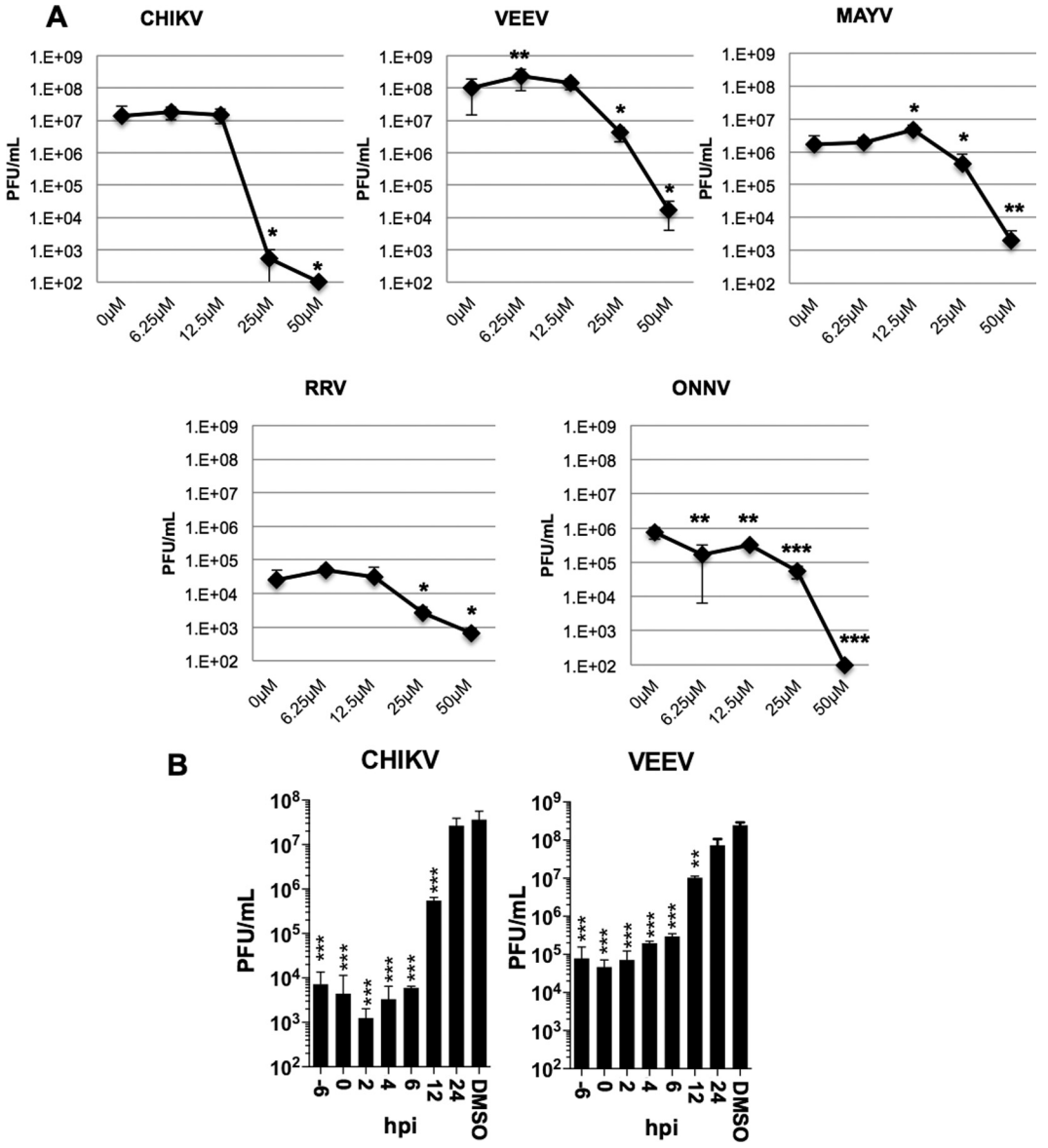

FIG 5 C11 generates a cellular state inhibitory to replication of multiple alphaviruses in a manner that correlates with molecular dose. (A) Average PFU per milliliter ± SD for CHIKV, VEEV, RRV, MAYV, and ONNV grown on THF cells in triplicate in the presence of the indicated concentrations of C11 added at 2 h preinfection. (B) Average PFU per milliliter ± SD for CHIKV and VEEV grown on THF cells in triplicate in the presence of 50 μM C11 added at the indicated hours postinfection (hpi). One-way ANOVA comparisons with Dunnett's *post hoc* test were made between C11- and DMSO-treated cells. **, $P < 0.01$; ***, $P < 0.001$.

displayed the largest titer drops in response to C11 and (ii) they represent both New and Old World phylogenetic clades. We treated THF-ISRE cells with 50 μM C11 −6, 0, 2, 4, 6, 12, or 24 h after infection with either CHIKV or VEEV and harvested the medium at 48 h postinfection for virus titration by plaque assay. As shown in Fig. 5B, the C11-associated significant inhibition of replication of both viruses was detectable when the compound was added at up to 12 h postinfection. This suggests that C11-induced innate factors are inhibitory to the growth of both virus types after virus-cell contact.

Antiviral effects of C11 require STING and IFNAR but not MAVS or TRIF. Examination of the molecular basis of C11-mediated innate immune induction, as defined by IRF3 activation, IFN secretion, and ISG transcription, revealed that STING, but not TRIF or MAVS, was essential to this process. Based on this, we hypothesized that only STING would be required for the observed antiviral activity associated with the

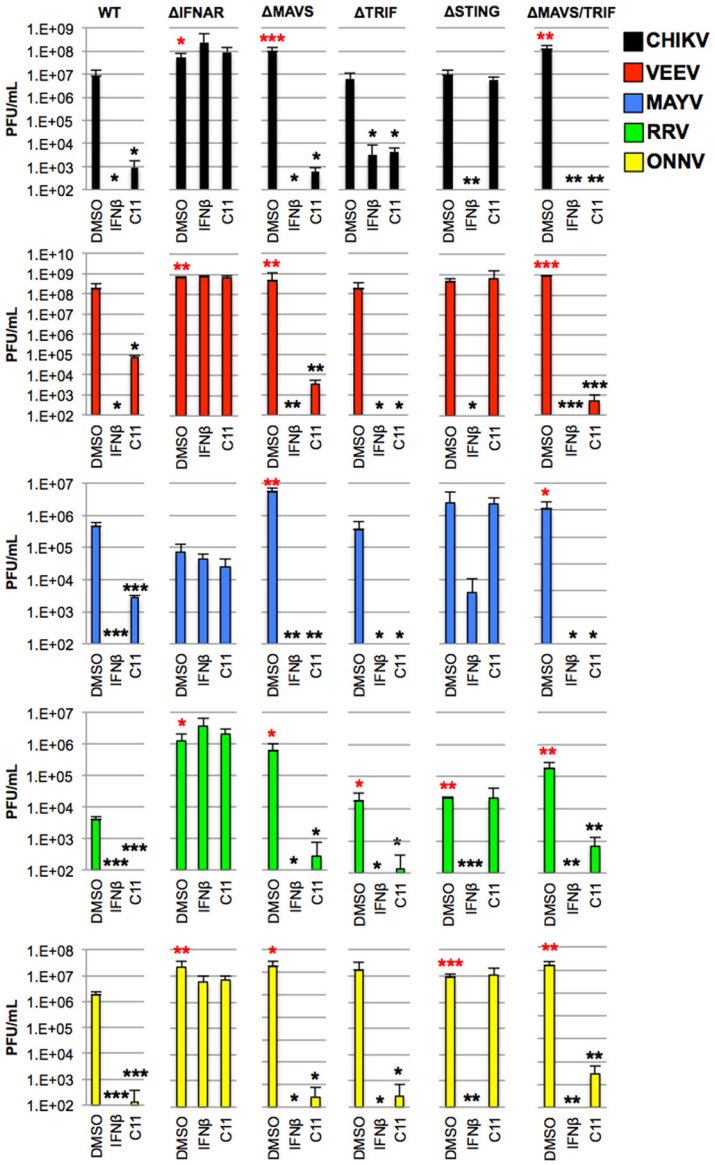

FIG 6 C11-mediated antiviral state is abrogated in cells lacking STING and IFNAR but not in those lacking MAVS or TRIF. The graphs show average PFU per milliliter ± SD at 48 h postinfection for CHIKV, VEEV, RRV, MAYV, and ONNV grown in triplicate on THF cells lacking the indicated proteins and pretreated for 6 h with 1% DMSO, 1,000 U/ml IFN-β, or 50 μM C11. Within cell types, statistical significance was determined by one-way analysis of variance with Dunnett's multiple-comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The significance of differences between WT and knockout cells for 1% DMSO treatment was similarly examined and is indicated with red asterisks.

molecule. Additionally, in the absence of IFN-dependent signaling, IRF3 has been shown to be required for the expression of a subset of ISGs that confer antiviral effects (53–55). Since C11 induced both IRF3 activation and IFN secretion, we therefore also asked whether the C11-mediated antiviral state required IFN-dependent JAK/STAT signaling. To address these questions, we employed THF cells lacking MAVS, TRIF, STING, MAVS/TRIF, or the type I IFN receptor (IFNAR) (18). We exposed these to 1% DMSO, 1,000 U/ml IFN-β, or 50 μM C11 for 6 h prior to infection. This concentration of C11 represents approximately 3 times the compound's EC_{90} for VEEV and CHIKV, 2.65 times the EC_{90} for ONNV, 2 times the EC_{90} for MAYV, and 2.22 times the EC_{90} for RRV.

As shown in Fig. 6, IFN-β generated a strong antiviral state in all cells except those lacking IFNAR, demonstrating that the JAK/STAT signaling pathway is intact except when the receptor is not present. Next, conditions inhibitory to the growth of all viruses

were observed in response to C11 in cells lacking MAVS, TRIF, or both proteins, indicating that these pathways are not essential to the establishment of the antiviral effect, in agreement with molecular observations regarding innate activation by the molecule (Fig. 3 and 4). Also, consistent with these results, cells lacking STING failed to inhibit alphavirus replication in response to 50 μM C11 (Fig. 6). Interestingly, the antiviral effects of C11 were also abrogated in the absence of IFNAR, suggesting that these were conferred (perhaps solely) by type I IFN secreted in response to the compound.

To assess the degree to which replication of the different virus types is affected by infection-induced type I IFN responses as well as the functions of the various adaptor proteins, we examined differences in viral titers between the wild-type cells and the described knockouts following control DMSO treatment from this experiment. As shown in Fig. 6, all viruses exhibited significantly higher titers in both cell types lacking MAVS, an observation consistent with an inhibitory impact of the IFN-inducing activity of the MAVS-dependent PRRs RIG-I and MDA5 on innate detection of viral RNA during alphavirus infection (34, 36, 37). Consistent with this, cells lacking IFNAR also displayed significantly higher replication of CHIKV, VEEV, RRV, and ONNV, suggesting that type I IFN signaling contributes substantially to the innate control of these viruses. Oddly, MAYV titers appeared to be lower in cells lacking IFNAR. Whether this is related to more rapid virus-induced cell death due to a lack of innate immune control in these cells, and thus a shorter duration of active virus production, would require more detailed investigation. Interestingly, the lack of STING and TRIF led to increased titers of RRV, and STING's absence also allowed ONNV to grow to significantly higher titers. Whether infection with these viruses triggers pathways dependent on TRIF or STING that then generate antiviral cellular states to which they are susceptible is a question outside the scope of this study. Overall, these results indicate that MAVS plays a crucial role in the innate intracellular response to infection with multiple alphaviruses that is likely mediated through infection-associated IFN secretion and IFNAR signaling.

DISCUSSION

Here we describe C11, a novel small molecule identified through a high-throughput screening endeavor that activates innate immune signaling in human cells. This response includes synthesis and secretion of type I IFN as well as expression of ISGs known to confer direct antiviral effects. The molecule is active on fibroblasts, primary PBMC, and myeloid-derived MonoMac 6 cells and exhibits insignificant cytotoxic effects at concentrations that induce innate activity. The signaling pathway stimulated by C11 terminates in IRF3 activation, but no clear evidence of NF-κB-dependent transcription was observed. Cells exposed to C11 exhibited a phenotypic state that was refractory to replication of multiple alphavirus types in a manner that required both the adaptor protein STING and IFNAR. Additionally, C11 was able to inhibit virus growth when added at up to 12 h postinfection, suggesting some therapeutic efficacy.

C11 was originally identified and subsequently validated as effective on telomerase-transduced human fibroblasts (Fig. 1B). Since these cells merely have extended life via telomerase and are not transformed via mutagenesis, they represent a suitable model of the innate immune signaling that is often defective in cancerous cell lines (56). Importantly, type I IFN responses are fully functional in these cells (57). Furthermore, their prolonged life span renders them tractable to transgenic manipulations, such as gene knockout or protein overexpression, that rely on multiple passages in the presence of selecting agents. Finally, they also strongly support growth of alphaviruses (34, 43) and are thus appropriate for studying antiviral phenotypes. While examining whether the compound was efficacious on other human cell types, we measured innate activation in the myeloid cell lines MM6 and THP-1. Surprisingly, ISRE-dependent transcription was observed in MM6 but not THP-1 cells (Fig. 1). The mechanism underlying this disparity is presently unclear but may be causally related to the STING allele observed in THP-1 cells (58), which differs from the homozygous WT genotype exhibited by THF and MM6 cells (not shown) and is known to exhibit decreased

reactivity to canonical ligands of the protein (59). A transcriptomic comparison of these cell types may also reveal potential STING-associated signaling components that differ between them and may play crucial roles in C11-induced activity. It was additionally of interest whether the molecule elicited innate activity in cells of murine origin given the power of *in vivo* mouse models available for alphavirus study (reviewed in reference 60). Unfortunately, treatment of murine RAW264.7 cells with C11 did not lead to detectable ISRE-dependent LUC activity (Fig. 1C). Whether this is due to the specificity of C11 for a human-specific, STING-inclusive pathway will require more exploration.

In an effort to understand the cellular signaling components required for C11-associated stimulation, we first examined endogenous mRNAs synthesized in response to C11 treatment. Transcripts known to be induced by IRF3-dependent processes, such as IFN-β, IFIT1, and Viperin, were also induced by C11, consistent with the transcription factor itself being activated by the compound (Fig. 2). Surprisingly, while stimuli that lead to IRF3 activation often also activate NF-κB, we failed to observe transcription of the genes for IL-1β (61), IL-6 (62), or IL-8 (63), which are conventional genes dependent on this transcription factor. We therefore targeted phosphorylation of IRF3 as a primary phenotypic readout to investigate C11-induced innate stimulation. As expected, the compound triggered phosphorylation of IRF3 S386 via a canonical TBK1-dependent mechanism (Fig. 3B). This response is known to occur by way of three distinct pathways, defined by the adaptor proteins MAVS, TRIF, and STING (15). We therefore employed loss-of-function assays using THF cell lines in which each of the proteins was singly deleted by use of CRISPR/Cas9 technology (18, 43). This revealed that C11 was only inactive in cells lacking STING (Fig. 3). However, we could not conclude from this result whether STING was required in combination with either MAVS (51) or TRIF (52), two scenarios that have both been described previously. To address this, we constructed THF cells that lacked both TRIF and MAVS but encoded STING. In these cells, C11 retained its stimulatory properties, indicating that the innate reaction to the compound requires STING and functions in the absence of both the TRIF and MAVS adaptor proteins (Fig. 4).

The unique essentiality of STING for C11-mediated innate activity is an important mechanistic attribute definitive of the molecule's mode of action. However, the actual molecular basis of STING-dependent IRF3/IFN induction in response to C11 may involve any number of intracellular targets and physiological apparatuses. For instance, small molecules that are direct activating ligands have been described for mouse STING isoforms (64–66) but, thus far, not for human orthologs. We asked whether C11 binds directly to the STING C-terminal (cytoplasmic) domain (CTD) in an *in vitro* assay by purifying the CTD of the protein and utilizing a fluorescence-based protein thermal shift assay (67). As shown in Fig. 3E, incubation of the protein with C11 failed to increase its thermal stability, in contrast to incubation with the canonical STING ligand 2′3′-cGAMP. While this result is inconsistent with direct engagement of the compound with the STING ligand binding domain, it does not formally rule out whether C11 stimulates the protein through its transmembrane domain by using an unknown activation mechanism. In addition, it is also possible that interaction between C11 and STING requires posttranslational modifications to the protein that occur in mammalian cells but not in bacteria used to synthesize the protein, and thus that compound-associated thermal shifts are not seen in our assay.

PRRs that require STING for IRF3-terminal signaling have been described before, including cGAS (68), IFI16 (69), and DDX41 (70). To determine whether any of these are essential to C11-mediated activity, we used CRISPR/Cas9 technology to construct THF-ISRE cell lines lacking each. Figure 3F illustrates that the C11-induced ISRE-LUC signal is substantial in each of these cell lines, suggesting that the molecule activates STING-dependent processes in a manner that does not require any of these PRRs, at least individually. It is also possible that C11 acts by way of regulatory cellular proteins associated with STING activity. This includes multiple negative regulators, including ULK1 (71), NLRX1 (72), and NLRC3 (73), that may represent inhibitory targets of C11. The compound may also engage an as yet unknown receptor or signaling molecule that

leads to STING activation. The identity of key host cell targets of C11 may actually be ascertainable by way of our observation that the compound induces ISRE signaling in MM6 but not THP-1 myeloid-derived cells (Fig. 1). Transcriptomic or proteomic contrasts between these cells may lead to identification of factors that are expressed in responsive MM6 cells but lacking in THP-1 cells. These could then be explored as potential targets by loss-of-function approaches with MM6 cells (e.g., CRSIPR/Cas9) or gain-of-function approaches with THP-1 cells.

Intriguingly, although C11 activates STING-dependent signaling, as does the canonical ligand 2′3′-cGAMP, surprising phenotypic differences were observed in cells treated with the two stimuli. For example, 2′3′-cGAMP-induced expression of mRNAs for IFIT1 and Viperin, but not IFN-β, was strongly enhanced over that with other IRF3-activating stimuli, including C11, in fibroblasts (Fig. 2A). Moreover, 2′3′-cGAMP was also able to trigger high levels of mRNA for IL-6, but not IL-1β or IL-8, in these cells, in contrast to C11, which induced none of these transcripts (Fig. 2B). These observations are consistent with C11 and 2′3′-cGAMP inducing fundamentally different innate signaling pathways, and perhaps transcription factors, in these cells. For example, the IL-6 promoter contains binding sites for multiple transcription factors, including AP-1, cyclic AMP-responsive element, CCAAT enhancer binding protein β, and NF-κB (74). It is possible that STING activation via 2′3′-cGAMP elicits a quantitatively or qualitatively different combination of these factors that manifests as differential gene transcription. It is also notable that C11 does not induce transcription of NF-κB-dependent genes that conventionally are believed to contribute to proinflammatory tissue states (75), suggesting that the compound may represent a more tolerable therapeutic. Understanding the molecular basis of these disparities would likely also shed light on the divergent and complex roles of STING-dependent IRF3 and NF-κB activation (76, 77).

Since C11 elicits the synthesis of type I IFNs (Fig. 2) as well as ISGs (Fig. 2 to 4), we predicted that it would correspondingly generate a cellular state unsupportive of virus replication. We chose to examine various alphavirus types for which human emergence events are documented and thus an unmet clinical need exists. The fact that members of this genus exhibit high sensitivity to type I IFNs (reviewed in references 40 and 42) provides an additional rationale for the utilization of alphaviruses. As shown in Fig. 5, preexposure of wild-type cells to C11 led to multilog reductions in the titer levels of CHIKV, VEEV, MAYV, RRV, and ONNV, with CHIKV exhibiting the largest overall titer decrease. Interestingly, while C11 was able to significantly inhibit the growth of RRV, the overall titer reduction was small, likely due to lower permissivity of fibroblasts to RRV replication. Data presented in Fig. 6 support that this is related to anti-RRV effects conferred by the type I IFN response, since RRV growth was drastically higher in cells lacking an RNA-dependent IFN-inducing (MAVS) or IFN signaling (IFNAR) pathway. The loss of C11-mediated antiviral activity in cells that lack STING (but not MAVS, TRIF, or both MAVS and TRIF) is consistent with the molecular observations indicating that STING is the only canonical adaptor protein involved in the C11-induced signaling pathway required for IRF3 activation and ISG expression (Fig. 3 and 4). Furthermore, the compound's antiviral effects were also lost when IFN-mediated JAK/STAT signaling was absent due to IFNAR deletion (Fig. 6). Collectively these results indicate that C11 triggers expression of type I IFN via a STING-dependent process that leads to the generation of an IFNAR-mediated antiviral cellular state. Though not specifically addressed here, it is reasonable to presume that this results from the activities of effector proteins encoded by ISGs (78). This result also points to a negligible or absent antiviral role for the subset of ISGs that can be expressed in an IRF3-dependent, IFN-independent manner (53, 54, 79).

An additional finding of this investigation involved the relevance of IRF3-terminal adaptor proteins and the alphavirus-induced IFN response to replication of these viruses. Relative to parental wild-type cells, all virus types grew to higher titers in cells lacking MAVS, the adaptor essential to IFN induction triggered in response to infection with RNA viruses, including alphaviruses (34, 36, 37). This result is consistent with a crucial antiviral role for this signaling pathway, likely via autocrine/paracrine IFN

signaling as well as a likely IFN-mediated positive-feedback loop that activates transcription of antiviral genes as well as the MAVS-dependent PRRs RIG-I (80) and MDA5 (81), both of which are crucial for detecting RNA viruses. Ultimately, autocrine IFN signaling can reduce viral replication in infected cells, and paracrine signaling can establish an antiviral state in uninfected cells. In further support of this, cells lacking IFNAR also allowed enhanced replication of CHIKV, VEEV, RRV, and ONNV, a result that parallels what has been seen for CHIKV growth in mice that are similarly deficient (82, 83). Unexpectedly, MAYV replication was not higher in THF-ΔIFNAR cells. It is possible that the absence of IFN-dependent activity is responsible for more uncontrolled virus replication and cell-to-cell spread, and consequently more rapid cytotoxicity and thus lower extracellular virus levels, at this time point (31, 40, 42, 84). Unfortunately, little is known about Mayaro virus innate sensitivity and pathogenesis, and alternative experimental conditions, such as an infection time course or kinetic monitoring of cell viability during infection, will be needed to understand this observation. An additionally intriguing result was related to the enhancement of the growth of RRV on cells lacking either STING or TRIF and the enhancement of that of ONNV on cells lacking STING. Since TRIF is downstream of the dsRNA PRR TLR3 (85), it is possible that the pathway is activated during RRV infection in a manner that elicits antiviral effects, although why this was observed only for this virus will require deeper exploration. STING can be activated by viral nucleic acid-independent signaling during RNA virus infection, including following virus-cell membrane fusion (86) and in response to mitochondrial disruption (87). Whether these or other STING-inducing processes are triggered during RRV or ONNV infection, ultimately leading to antiviral effects to which these viruses are susceptible, is a possible scenario that warrants examination.

Pharmacologic activation of STING-dependent signaling has shown promise in diverse clinically impactful applications, including broad-action antiviral treatments (19, 23, 43, 88), vaccine adjuvants (89), and immunogenic tumor clearance (90). This has led to academic and commercial efforts to formulate CDNs for pharmaceutical use, including their advancement to an ongoing clinical trial (NCT02675439). Unfortunately, CDNs may be chemically undesirable for research and clinical work because (i) they violate the Lipinski rules (91) for druglikeness and are not amenable to large structural changes, (ii) they are susceptible to phosphodiesterase-mediated degradation (92), and (iii) their size and hydrophilicity render them unable to permeate cell membranes (93). Small-molecule STING activators mitigate these factors, as well exemplified by the mouse-specific compound DMXAA (64, 65, 94). While other small molecules have been shown to induce STING-dependent activity (19, 43, 66, 88), to our knowledge, none work in both mice and humans. Novel small-molecule STING agonists that are efficacious across species are thus highly sought, as they may lead to valuable research tools for work toward understanding STING-mediated processes as well as clinically valuable drugs, and their use in animals enables broad assessments of safety and biological mechanisms. Our group conducted a high-throughput screen to identify novel compounds that activate IRF3/IFN-dependent processes in human cells. This led to discovery of C11, a molecule that triggers IRF3/IFN-dependent responses in a manner that requires STING. To identify a fundamental pharmacophore that is required for the compound's innate activating function, we constructed a group of C11 analogs to facilitate characterization of structure-activity relationships (Fig. 1E). Unfortunately, all chemical modifications abrogated the molecule's ability to induce ISRE-LUC expression. As such, additional analogs, perhaps displaying more subtle alterations, will be necessary.

In conclusion, we have successfully identified a novel small-molecule agonist of the STING pathway that is capable of inducing expression of type I IFNs as well as antiviral effector gene products in human cells via IRF3 phosphorylation. Using a CRISPR/Cas9-driven reverse genetics approach, STING, but not TRIF or MAVS, was shown to be required for establishment of an IFN-dependent antiviral state in cells that is effective against multiple emerging alphavirus types. These findings further support the potential therapeutic role for small-molecule agonists of the STING pathway in a number of

diseases, including virus infections and cancers as well as others that have yet to be identified.

MATERIALS AND METHODS

Reagents and antibodies. Dimethyl sulfide (DMSO) was purchased from Thermo Fisher. Human recombinant IFN-β was obtained from PBL. Lipopolysaccharide (LPS) was obtained from Sigma. 2′3′-cGAMP was purchased from Invivogen. Stocks of C11 were originally obtained from Enamine and resynthesized in larger quantities by the OHSU Medicinal Chemistry Core Facility. Puromycin was obtained from Invivogen and used at 3 μg/ml in resistant cell culture. G418 sulfate was obtained from Enzo Life Sciences and used at 400 μg/ml in resistant cell culture. One-Glo cell lysis/luciferin and CellTiter-Glo viability assays were obtained from Promega. Lipofectamine 3000 was obtained from Invitrogen. Sources and concentrations of antibodies used against the following antigens are indicated in parentheses: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (SC-51906; Santa Cruz) (1:10,000), IRF3 (4302; Cell Signaling) (1:1,000), phospho-IRF3 (4947; Cell Signaling) (1:1,000), Mx1 (GTX11153; Gentex) (1:2,000), ISG15 (200-401-438; Rockland) (1:2,000), cGAS (15102; Cell Signaling) (1:1,000), DDX41 (15076; Cell Signaling) (1:1,000), and IFI16 (14970; Cell Signaling) (1:1,000).

Cell and virus cultures. Human foreskin fibroblasts, originally obtained from the American Type Culture Collection, were stably transduced with constitutively expressed human telomerase reverse transcriptase and the IRF3/IFN-responsive pGreenFire-ISRE lentivector (System Biosciences), sorted, and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml), HEPES (10 mM), and G418 (400 μg/ml). MonoMac6 (MM6) cells were a kind gift from Michael Gale (University of Washington) and were transduced with a lentivector containing the pGreenFire ISRE cassette. Transduced MM6 cells were then maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 U/ml), HEPES (10 mM), and G418 (400 μg/ml) at a density of 3×10^5 to 10×10^5 cells/ml. THP-1 ISG-lucia cells were purchased from Invivogen. THP-1 ISG-lucia cells were differentiated by 2 h of treatment with 100 ng/ml PMA, and then the PMA was removed and replaced with complete medium for 72 h of incubation prior to all assays. Vero cells were obtained from Alec Hirsch (Oregon Health & Sciences University) and were grown as described previously (34). Human peripheral blood mononuclear cells were obtained from StemCell Technologies and maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 U/ml), and HEPES (10 mM). All cells were grown at 37°C and 5% CO_2. Sendai virus (SeV) was obtained from Charles River Laboratories and used at 160 hemagglutination units (HAU)/ml. Human cytomegalovirus was grown, titrated, UV inactivated (4 times for 30 s each; 600 kJ), and exposed to cells at a multiplicity of infection (MOI) of 3 (pre-UV irradiation). Chikungunya virus (CHIKV) was prepared as previously described (18, 43). Venezuelan equine encephalitis virus strain TC83 (VEEV), Mayaro virus (MAYV), Ross River virus (RRV), and O'nyong-nyong virus (ONNV) were all obtained from Robert Tesh (University of Texas). All viruses were propagated on C6/36 mosquito cells (in DMEM-10% FBS at 28°C) for 48 h prior to ultracentrifugation, and their titers were determined by serial dilution plaque assay on Vero cells. All infections were carried out following 2 h of pretreatment (except where otherwise noted) with C11, IFN-β, or DMSO at the indicated concentrations, and cells were infected at an MOI of 1 PFU per cell. 2′3′-cGAMP was transfected into cells by use of Lipofectamine LTX following the manufacturer's protocol. C11 was added directly to low-serum cell culture medium (2% FBS) for all THF cell treatments or to X-Vivo15 defined-serum medium (0% FBS) for all MonoMac6 cell treatments.

CRISPR/Cas9-mediated genome editing. Genome editing using lentivector-mediated delivery of CRISPR/Cas9 components was performed generally as described previously (18, 43). For simultaneous deletion of TRIF and MAVS, we modified the lentiCRISPRv2 vector (AddGene) (95, 96) by replacing the puromycin resistance open reading frame (ORF) with one that confers hygromycin resistance. TRIF-specific guide RNA (gRNA) was cloned into this vector, which was then used to transduce THF-ISRE-ΔMAVS cells. The double transfectant was then selected for resistance, serially diluted, and validated for the knockout as described previously (18, 43). Sequences of new gRNA targets used here are as follows: for DDX41, TGGAGGAGTCGGAACCCGAA; for IFI16, TATACCAACGCTTGAAGACC; and for cGAS, GAACTTT CCCGCCTTAGGCA. Lentivirus was made by transfecting the lentivector along with a packaging plasmid (psPAX2; AddGene) and a vesicular stomatitis virus G protein pseudotyping plasmid (pMD2.G; Addgene) into Lenti-X 293T cells (Clontech) by use of Lipofectamine-LTX (Life Technologies, Inc.). Medium was harvested at 48 h and 72 h posttransfection, centrifuged (3,000 $\times$ *g* for 10 min), and filtered through a 0.45-μm filter to remove cell debris. Subconfluent target cells were exposed to lentivirus for 8 h in the presence of 5 μg/ml Polybrene. After target cells reached confluence, cultures were split into DMEM plus 10% FBS containing 3 μg/ml puromycin or 100 μg/ml hygromycin. Transduced cells were passaged in the presence of selecting agents for 7 to 10 days before protein knockout was examined by immunoblotting. Cells were next serially diluted twice in 96-well plates to obtain oligoclonal lines purified for gene deletion. Protein knockout was additionally verified functionally by measuring phenotypic responsiveness to appropriate stimuli.

Luciferase reporter assay and type I interferon bioassays. For direct THF-ISRE cell reporter assays, confluent cells were plated at 20,000 cells/well in a white 96-well plate 24 h before stimulation. Treatments were performed in quadruplicate in 40 μl of either DMEM plus 2% FBS (THF cells) or X-Vivo15 medium (MM6 cells) for 8 h, unless otherwise indicated. Steady-Glo lysis/luciferin reagent (Promega) was added (1:1 [vol/vol]) to each well, and luminescence was measured on a Synergy plate reader (BioTek). For cell viability assays, CellTiter-Glo reagent was used following the manufacturer's suggested protocol. For type I IFN bioassays, cells of interest were plated at 50,000 cells/well in 24-well plates and serum starved in either DMEM plus 2% FBS (THF cells) or X-Vivo15 medium (MM6 cells) for 1 h prior to

treatment. After treatment for 24 h, the medium was harvested and clarified at 10,000 × *g* for 3 min. Recombinant IFN-β (at 40, 20, 10, 5, 2.5, 1.25, and 0.63 U/ml) was used to generate a standard response curve. The supernatant or standard was then added to THF-ISRE-ΔIRF3 cells (do not respond to STING/IRF3-inducing stimuli) plated as described above for 8 h, and luminescence was measured. IFN was quantitated by curve fitting relative to the signals generated from the standards.

Immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as follows. After trypsinization and cell pelleting at 2,000 × *g* for 10 min, whole-cell lysates were harvested in RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher). Lysates were electrophoresed in 8% polyacrylamide gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) by semidry transfer at 200 mA for 35 min. The blots were blocked at room temperature for 2 h or overnight, using 5% nonfat milk in 1× phosphate-buffered saline (PBS) containing 0.1% Tween 20. The blots were exposed to primary antibody in 5% nonfat milk in 1× PBS containing 0.1% Tween 20 for 18 h at 4°C. The blots were then washed in 1× PBS containing 0.1% Tween 20 for 20, 15, and 5 min, followed by deionized water for 5 min. A 1-h exposure to horseradish peroxidase-conjugated secondary antibodies and subsequent washes were performed as described for the primary antibodies. Antibodies were visualized using enhanced chemiluminescence (Pierce).

RNA isolation and semiquantitative reverse transcription-PCR. Total RNA was isolated from cells, treated with the DNase provided in a DNA-free RNA isolation kit (Zymo Research) according to the manufacturer's protocol, and quantified by using UV spectrometry. Single-stranded cDNA for use as a PCR template was made from total RNA and random hexamers to prime first-strand synthesis via a RevertAid First Strand cDNA synthesis kit (Thermo Fisher) per the manufacturer's protocol. Comparison of mRNA expression levels between samples was performed using semiquantitative real-time reverse transcription-PCR (qPCR) with an Applied Biosystems sequence detection system according to the $\Delta\Delta C_T$ method (97), with GAPDH as a control. Prevalidated Prime-Time 6-carboxyfluorescein qPCR primer/probe sets obtained from IDT were used for all genes.

STING protein purification and thermal shift assays. Assays of the molecular interaction between the purified human STING C-terminal domain (CTD) (amino acids 137 to 379; nontransmembrane domain) and C11 were performed as previously reported (43). Briefly, the 6×His STING CTD open reading frame was cloned into pRSET-B (Invitrogen) and used to transform the *Escherichia coli* strain pLysS (Promega). The transformed *E. coli* cells were then induced to express the 6×His STING CTD by use of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 16°C for 18 h. The STING CTD was purified by nickel-affinity chromatography (Clontech Laboratories) and then further purified by gel filtration chromatography (HiPrep 16/60 Sephacryl S-100 HR column; GE Healthcare Life Sciences). Eluted proteins were concentrated using Amicon centrifugal filters (10-kDa cutoff). For thermal shift assays, SYPRO Orange dye was used, following the manufacturer's suggested protocol, to determine protein stability in the presence and absence of 2′3′-cGAMP (Invivogen) or C11, as previously described (43).

Statistical analyses. Unless otherwise noted, all data analyses were performed in Prism 6.0, using a two-tailed independent *t* test or one-way analysis of variance (ANOVA), with Bonferroni or Dunnett's *post hoc* test where indicated. All group variances were confirmed to be nonsignificant by the Brown-Forsythe test ($P \geq 0.05$). Regression analyses of known values of IFN-β to interpolate unknown values in IFN bioassays were performed using 4-parameter logistic nonlinear regression analyses and confirmed to give an appropriate fit ($r^2 \geq 0.90$).

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