Mouse, but not Human STING, Binds and Signals in Response to the Vascular Disrupting Agent 5,6-Dimethylxanthenone-4-Acetic Acid


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Over the past several years, attention has focused on the role of the innate immune system in both pro- and antitumor immunity (1, 2). Manipulating immune surveillance and effector mechanisms is important for antitumor immunity. Vadimezan or ASA404 (originally called 5,6-dimethylxanthene-4-acetic acid [DMXAA]) is a xanthenone derivative with potent antitumor effects in multiple mouse models (3). In addition to disrupting tumor blood supply, the antitumor effects of DMXAA result from the activation of NK cells and the release of cytokines from tumor-associated macrophages leading to hemorrhagic necrosis in tumors (4, 5). Additionally, production of chemokines such as MCP-1, IFN-γ-inducible protein (IP)-10, and RANTES leads to the recruitment of activated tumor-specific CD8+ T cells that contribute to the disruption of tumors. DMXAA is also a potent inducer of IFN-β (5–7), a cytokine typically induced during infection with viral and bacterial infections. The induction of IFN-β expression by DMXAA slows the growth of tumors in vivo (8, 9). DMXAA showed great promise in initial phase clinical trials (10, 11) but ultimately performed poorly in follow up phase III clinical trials. Understanding the mechanisms by which DMXAA elicits cytokine and IFN production could allow a better understanding of its antitumor effects and enable the development of improved antitumor agents.

DMXAA treatment of macrophages has been linked to MAPK and NF-κB signaling (12–16) although activation of these pathways is very modest (6). In contrast, we identified IFN regulatory factor 3 (IRF3), a transcription factor important in innate immunity as an important mediator of DMXAA-induced macrophage activation (6). DMXAA is a very strong activator of IRF3 signaling. Normally, IRF3 is present in the cytoplasm and undergoes phosphorylation leading to its dimerization and interaction with the coactivators CBP-p300. TANK-binding kinase 1 (TBK1), an IκB kinase–related kinase, coordinates the phosphorylation-induced activation of IRF3 leading to transcriptional regulation of immune response genes including type I IFNs and antiviral IFN-stimulated genes (17–19). Despite more than 15 years of research on DMXAA and its parent compound, flavone acetic acid (FAA), the molecular mechanism responsible for the immune stimulatory effect of DMXAA remains unknown.

Great progress has been made over the past decade in understanding TBK1 activation (20, 21). Several classes of innate sensors including the TLRs and retinoic acid–inducible gene 1 (RIG-I)–like helicases engage TBK1–IRF3 signaling pathways to regulate transcription of type I IFNs. Activation of TBK1 by DMXAA occurs independently of TLRs and RIG-I–like receptors (6). DNA sensing was a very strong activator of IRF3 signaling. Normally, IRF3 is present in the cytoplasm and undergoes phosphorylation leading to its dimerization and interaction with the coactivators CBP-p300. TANK-binding kinase 1 (TBK1), an IκB kinase–related kinase, coordinates the phosphorylation-induced activation of IRF3 leading to transcriptional regulation of immune response genes including type I IFNs and antiviral IFN-stimulated genes (17–19). Despite more than 15 years of research on DMXAA and its parent compound, flavone acetic acid (FAA), the molecular mechanism responsible for the immune stimulatory effect of DMXAA remains unknown.
receptors such as DAI, IFI16, DDX41, and most recently cGAS have all been shown to couple dsDNA recognition to TBK1 activation (22–28). An endoplasmic reticulum and/or mitochondrial resident protein called stimulator of IFN gene (STING) is a critical mediator of DNA-induced TBK1 activation (29–32). In addition to this adaptor-like function for STING, STING also acts as a direct innate immune sensor of cyclic di-guanosine monophosphate (c-di-GMP) and cyclic diadenosine monophosphate (c-di-AMP), conserved signaling molecules produced by bacteria (33).

Using RNA interference in macrophages, as well as using macrophages from mice lacking STING, we recently found that DMXAA-induced IFN production required STING (34). In this study, we sought to delineate the molecular mechanism of activation of STING by DMXAA. We found that DMXAA directly binds to STING and activates the TBK1–IRF3 signaling pathway resulting in IFN-β production. Ectopic expression of STING in 293T cells which, themselves are unable to respond to DMXAA, facilitated DMXAA-induced TBK1 activation, IRF3 phosphorylation, and IFN-β gene induction. STING bound DMXAA directly via its C terminal domain. Remarkably, the ability of STING to mediate DMXAA signaling was seen only with murine STING. When human PBMC or human THP1 cells were treated with DMXAA, no induction of type I IFNs or IFN-stimulated genes was observed. Consistent with these observations, human 293T cells expressing human STING or a chimeric molecule in which the C-terminal domain (CTD) of murine STING was replaced with that of human STING failed to respond to DMXAA. Unlike murine STING, which efficiently binds DMXAA, human STING failed to bind DMXAA. Similarly, comparison of DMXAA with cyclic dinucleotides revealed important differences between human and mouse STING for cyclic–dinucleotide signaling. Taken together, these findings detail a STING pathway critical for DMXAA signaling and reveal a likely explanation for the failure of this molecule to exert its antitumor effects in humans.

Materials and Methods

Abs and reagents

DMXAA was from Sigma-Aldrich (D5817). Cyclic-di-GMP was from Biolg. The phospho-TBK1 (Ser 172) Ab used on the TBK1 immunoprecipitations (IPs) was from BD Biosciences (558397). The total TBK1 Ab used on the TBK1 IPs was from Imgenex (IMG-139A). Anti-Flag agarose beads (A2220), mouse M2 anti-Flag (F3165), rabbit anti-Flag (F7425), and mouse anti–β-actin (A5316) were from Sigma-Aldrich. Phospho-TBK1 mSer172 (5483), total TBK1 (3504), and phospho-IRF3 mSer 396 (4947) Abs were from Cell Signaling Technology. STING-deficient mice were from G. Barber (University of Miami, Miami, FL).

Plasmids

pOTB7-human STING (hSTING) (Openbiosystems), pUNO-hSTING (Invivogen), and pCMV-Sport6-mouse STING (mSTING) (Tm Reva, 73) (Openbiosystems) were all subcloned into pEF-Flag-His. pEF-Bo hSTING with Arg and His at position 232 behaved similarly in all of our DMXAA and cyclic dinucleotide assays. pEF-Bo hSTING-Flag-His was generated by using the hSTING N-terminal forward primer and the hSTING C-terminal reverse primer to PCR amplify hSTING cDNA (aa 1–379). The resulting PCR product was digested with Esp3I to generate XhoI and BamHI-compatible ends. The Esp3I-digested PCR product was ligated into BamHI–XhoI-digested pEF-BO Flag-His. pEF-BO mSTING-Flag-His was generated using the same strategy and the appropriate primers (mSTING N-terminal forward primer combined with mSTING C-terminal reverse primer using pCMV-Sport6-mSTING as template. Plasmids encoding MAVS were originally from Z. Chen (University of Texas Southwestern Medical Center, Dallas, TX). Human NOD2 was from Millennium Pharmaceuticals and murine NOD2 from M. Kellner (University of Massachusetts Medical School, Worcester, MA).

Generation of STING chimeras

Primers (forward, 5′-GCCCAGCAGCTCTCTGGACCACCATGGGCACCACCTCCAGGCTG-3′; reverse, 5′-ATCCGATCTCCTGACATCCATGGCAC-3′) were used to PCR amplify cDNA encoding hSTING N-terminal (aa 1–162). These primers introduce Esp3I sites at both ends of the cDNA. The forward primer also introduced an XhoI site immediately after the Esp3I site. Another pair of primers (forward, 5′-CATGGGCGCTCCTGGATATACATCGGATACGGA-3′; reverse, 5′-TTGGTACCGCTTCGAGAAGAAATCGCGTGCCGAGA-GGAGGCGCT-3′) was used to amplify hSTING C-terminal (aa 163–379). In a fashion similar to the primers for the N-terminal region of hSTING, these primers introduced Esp3I sites at either end of the cDNA. The reverse primer introduced a BamHI site immediately after the Esp3I site. The same strategy was used to PCR amplify mSTING N-terminal (aa 1–161; forward primer, 5′-TCCGGGCGGTCTCCTGAGAAGAAATCGCGTGCCGAGA-GGAGGCGCT-3′; reverse primer, 5′-CCCAATGTGCCGTAAGGCGCACGCA-3′; and reverse, 5′-CCCCAATGTGCCGTAAGGCGCACGCA-3′) and mSTING C-terminal (aa 162–378). For forward primer, 5′-CAGCGGCGCTTCGAGAAGAAATCGCGTGCCGAGA-GGAGGCGCT-3′ and reverse primer, 5′-TG- GTACCGCTTCGAGAAGAAATCGCGTGCCGAGA-GGAGGCGCT-3′). All four PCR products were digested with Esp3I. The hSTING N-terminal cDNA fragment and the mSTING C-terminal cDNA fragment were ligated into pEF-BOS Flag-poly His Digested with XhoI and BamHI. The resulting vector encodes the h-mSTING chimera with a C-terminal Flag-His tag. The mSTING N-terminal fragment and the hSTING C-terminal fragment were combined in a similar fashion to create pEF-BOS mSTING-Flag-His. The fusion site (Ser162/Tyr162–163) occurs within helix 1 of the cyclic-di-GMP binding domain, the long hydrophobic helix that forms a major part of the dimerization interface (6–10). This site was chosen because the cDNA sequence encoding these two amino acids is perfectly conserved between mouse and human, creating complementary overhangs after digestion with Esp3I.

Cell stimulations

Human embryonic kidney 293T cells were from American Type Culture Collection (Manassas, VA). PBMC were freshly isolated by density-gradient centrifugation using Ficoll Hypaque (GE Healthcare). Wild-type immortalized bone marrow–derived macrophages were generated as described previously (35). Bone marrow derived macrophages were isolated from mice as described previously (35). 293T cells, mouse macrophages PBMC and THP1 cells were stimulated with DMXAA essentially as described previously (1). Cells were transfected with c-di-GMP or cyclic-di-AMP using Lipofectamine 2000 (Life Technologies) as described previously (5).

Reporter assays

Reporter assays were carried out as described previously (4). Cells were transfected with reporters plus 10 or 50 ng of the indicated STING, DX41, NOD2, or MYD88. Eighteen hours after transfection, cells were stimulated with DMXAA (0.2, 1, 5, 25, or 50 μg/ml) for 6 h, and luciferase activity was measured.

In vitro kinase assays

Kinase assays were carried out as previously described (1–3), with modifications. Whole-cell lysates were prepared and endogenous TBK1 was immunoprecipitated using a polyclonal Ab (89246) raised to a C-terminal modifications. Whole-cell lysates were prepared and endogenous TBK1 was immunoprecipitated using a polyclonal Ab (89246) raised to a C-terminal modifications. Whole-cell lysates were prepared and endogenous TBK1 was immunoprecipitated using a polyclonal Ab (89246) raised to a C-terminal modifications.
the nCounter prep station, followed by quantification with the nCounter Digital Analyzer. We normalized the data obtained for each sample to the expression of six control mouse or human genes depending on the cell types used. For every sample, we computed the weighted average of the mRNA counts of the six control transcripts and normalized the sample’s values by multiplying each transcript count by the weighted average of the controls. Values were log-transformed and displayed via heat map generated using the ggplot package within the open source R software environment.

**Preparation of 293T cell lysates and immunoprecipitations**

HEK293T cells were plated at a density of 1 × 10⁶ cells/well in a 6-well plate. The following day, the cells were transfected as indicated with STING constructs using Lipofectamine 2000 (Invitrogen). The day after that, the cells were rinsed once with PBS and transferred to Eppendorf tubes in PBS containing 1 mM EDTA. The cells were pelleted briefly by centrifugation at 1000 x g at 4°C. The cell pellet was lysed in an equal volume of digitonin lysis buffer (0.5% digitonin, 20 mM Tris-HCl [pH 7.4], and 150 mM NaCl) containing protease inhibitors (Roche) for 10 min on ice. The cell lysates were centrifuged at 10,000 x g for 10 min at 4°C. The protein concentration in the resultant supernatant was measured using the Bradford reagent (Bio-Rad). The cell lysates were subjected to a c-di-GMP binding (cross-linking) assay (see below). The lysates were separated by SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane, which was then probed with rat anti-hemagglutinin (HA) Abs (Roche), to confirm STING–HA expression, and mouse anti-β-actin Abs (Santa Cruz Biotechnology). To immunoprecipitate HA-tagged STING, the cell lysates were prepared similarly in digitonin lysis buffer and incubated with anti–HA-Ab–conjugated agarose beads (Sigma-Aldrich) for 2 h at 4°C. Washed beads were subjected to a c-di-GMP binding assay or separated by SDS-PAGE and stained with colloidal blue protein stain (Thermo Scientific). In other assays, flag tagged STING was detected by immunoblotting with anti-Flag Ab.

c-di-GMP UV cross-linking assay

The c-di-GMP binding assay (also called the cross-linking assay) was carried out as described previously (33). Briefly, 50 μg HEK293T cell lysate at a final concentration of 2 μg/μl, or 1 μg recombinant His6-tagged STING, was incubated with 2 μCi radiolabeled nucleotide in binding buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, and 1 mM MgCl₂) for 15 min at 25°C. The reactions were irradiated at 254 nm for 20 min on ice at a 3 cm distance from a UVG-54 mineral light lamp (UVP). Immediately after crosslinking, the reactions were terminated by the addition of SDS sample buffer (40% glycerol, 8% SDS, 2% 2-ME, 40 mM EDTA, 0.05% bromophenol blue, and 250 mM Tris-HCl [pH 6.8]), boiled for 5 min, and then separated by SDS-PAGE. The gels were dried, exposed to a phosphor screen, and visualized using a Typhoon Trio imager (GE Healthcare).

**Thermal shift assay**

Both human and mouse STING CTDs were expressed and purified similar to what was reported (33). The thermal shift assay was performed with a ProteoStat thermal shift stability assay kit (Enzo Life Sciences, Farmingdale, NY) following the manufacturer’s instructions. Thirty-six micromoles of protein and 200 μM ligands were mixed and heated using a linear gradient of 20–80°C in 30 min. Fluorescence signal as a function of temperature was recorded using a RealPlex4 real-time PCR cycler (Eppendorf, Hauppauge, NY) with the excitation and emission wavelengths of 470 and 605 nm, respectively. Each sample was measured in triplicate and fitted with the Boltzmann equation using GraphPad Prism (GraphPad Software, San Diego, CA).

**Statistical analysis**

Data were analyzed by one-way or two-way ANOVA followed by the Bonferroni post hoc test using Prism software. The p values < 0.05 were considered significant.

**Results**

To study TBK1 activation in response to DMXAA treatment of murine bone marrow–derived macrophages, we isolated the TBK1 kinase complex using a highly specific anti-TBK1 Ab, and performed an in vitro kinase assay to monitor IRF3 phosphorylation. DMXAA induced rapid TBK1 activation resulting in phosphorylation of IRF3 as early as 15 min poststimulation (Fig. 1A). We
also observed rapid signal-induced phosphorylation of TBK1 in its activation loop (pS172) (Fig. 1A, lower panels) with similar kinetics. Because activation of TBK1 by DMXAA occurs independently of TLRs and RIG-I–like receptors (6), we wanted to examine the role of the STING pathway in DMXAA-induced TBK1 activation by examining the ability of DMXAA to activate TBK1 in wild-type macrophages and in macrophages from STING-deficient mice. The ability of DMXAA to induce TBK1 activation loop phosphorylation and to activate TBK1 kinase activity leading to IRF3 phosphorylation was entirely dependent on STING (Fig. 1B). STING-deficient macrophages displayed TBK1 and IRF3 activation normally following LPS stimulation. We have previously shown that STING-deficient macrophages are impaired in their ability to induce IFN-β expression following DMXAA treatment (34). To understand the immunostimulatory potential of DMXAA, we profiled the expression of a broad panel of inducible immune genes (type I IFNs, as well as a selection of inflammatory cytokines and IFN-stimulated genes [ISGs]) using a nonenzymatic RNA profiling technology that uses bar-coded fluorescent probes (nCounter, Nanostring). Using this technology, we found that DMXAA robustly activated expression of type I IFNs, IFN-stimulated genes as well as inflammatory cytokines, chemokines, and other immune regulators in wild-type cells. In all cases, induction of these genes was largely abrogated in macrophages from mice lacking STING (Fig. 1C). Notably, DMXAA induced IL-6, IL-1α, IL-12α, IL-1 receptor antagonist, TNF-α, and IL-21, as well as A20 in a STING-dependent manner. Because all of these genes are NF-κB–dependent these data indicate that in addition to activating the TBK-1 and IRF3 pathway, DMXAA also induced NF-κB target genes via STING. These data indicate that DMXAA signals via STING to induce both TBK1–IRF3-dependent IFN production and NF-κB–dependent cytokine gene transcription in mouse macrophages.

It is currently unclear how DMXAA engages the STING pathway. In an effort to understand these events further, we sought to identify molecules that could reconstitute the IFN response to DMXAA in 293T cells, which do not respond to DMXAA. We expressed mouse STING in 293T cells and monitored IFN-β luciferase reporter gene activation as well as TBK1–induced phosphorylation of IRF3. DMXAA induced IFN-β luciferase reporter gene expression, as well as IRF3 activation, only in cells expressing mouse STING. Low level of mouse STING protein was sufficient to reconstitute the responsiveness of 293T cells to DMXAA (Fig. 2A, 2B). Similar results were obtained when the IFN-β positive regulatory domain (PRD) III-I luciferase reporter, which contains a multimerized PRDIII-I element that binds to activated IRFs) was used (Fig. 2A, right panel). Recently, NOD 1 and NOD2 were shown to contribute to DMXAA-induced NF-κB signaling using HEK293 cells. NOD1 and NOD2 are members of the larger NOD-like receptor family and are important in recognizing specific motifs within peptidoglycans of both Gram-negative and Gram-positive bacteria. NOD1 and NOD2 signal via the downstream adaptor serine/threonine kinase receptor interacting protein-2 (RIP2/CARDIAK/RICK) to initiate NF-κB activation and the release of inflammatory cytokines/chemokines.

In light of these findings we next compared the ability of STING and NOD2 to reconstitute the responsiveness of 293T cells to DMXAA. In contrast to murine STING, neither human nor murine NOD2 reconstituted DMXAA-induced IFN-β luciferase reporter gene expression (Supplemental Fig. 1A). Although, both human and murine NOD2 constitutively increased basal NF-κB activation, similar to what we found with MyD88, neither human nor murine NOD2 reconstituted DMXAA-induced NF-κB activation. Under these conditions mouse STING facilitated a modest increase in NF-κB activation (Supplemental Fig. 1B).

c-di-GMP and c-di-AMP conserved signaling molecules produced by bacteria have been shown to signal via STING (33). Recently DDX41; a member of the DEXD helicase family has also been shown to be important for DNA and cyclic–dinucleotide signaling (37). We therefore also compared the ability of mouse STING and DDX41 to reconstitute the IFN response to DMXAA in 293T cells. Unlike mouse STING, however, ectopic expression of DDX41 did not reconstitute DMXAA signaling (Supplemental Fig. 1C). The ability of mouse STING in 293T cells to facilitate DMXAA-induced signaling is reminiscent of published studies with cyclic-di-AMP and cyclic-di-GMP (33). Collectively, these
results reveal that STING expression is sufficient to restore the responsiveness of 293T cells to DMXAA.

In the case of cyclic-di-nucleotides, STING acts as a sensor by directly binding these small molecules (33). STING encodes an N-terminal domain with multiple transmembrane segments, followed by a globular CTD. To examine whether STING can bind to DMXAA, we took advantage of an in vitro UV radiation cross-linking assay used previously to demonstrate cyclic-di-GMP binding to STING (33). Consistent with published studies (38–41), we observed cyclic-di-GMP binding to the mouse STING CTD after UV cross-linking in both 293T cell transfectants and when a purified C-terminal domain of mouse STING was used in vitro (Fig. 3A, 3B). In both cases, binding of cyclic-di-GMP to the STING CTD was specifically competed away with cold unlabeled c-di-GMP. We reasoned that if DMXAA similarly bound STING, it should also compete with radiolabeled c-di-GMP for STING binding. Similar to what we observed with cold c-di-GMP, binding of labeled c-di-GMP to STING was competed away with DMXAA (Fig. 3B, right panel). These data show that c-di-GMP and DMXAA compete for the mouse STING CTD binding indicating that STING is a direct sensor for DMXAA. We next examined the direct binding of DMXAA to the mouse STING CTD by using a thermal shift assay. This assay is used to monitor the thermal stability of proteins and investigate factors affecting this stability. Using this assay, we found that like c-di-AMP and c-di-GMP, DMXAA increased the melting temperature (Tm) of the STING CTD, an indication of binding and stabilization of the STING CTD structure (Fig. 3C). These data indicate that DMXAA, c-di-GMP and c-di-AMP all bind directly to mouse STING via its CTD.

While examining the immune stimulatory activity of DMXAA, we compared responses in human and mouse cells and, to our surprise, found that Unlike murine macrophages or dendritic cells (data not shown), PBMC isolated from normal healthy volunteers failed to induce measurable IFN-β responses to DMXAA. This was in contrast to PBMC treated with poly(deoxyadenylic-deoxycytidylic) acid (poly(dA-dT)), a synthetic B-form dsDNA that elicited a strong IFN-β mRNA induction (Fig. 4A). Surprisingly, there was also a very minimal response to c-di-AMP and c-di-GMP in human PBMC. Therefore, we extended this analysis to include a broader panel of inducible immune response genes, which were all robustly induced by poly(dA-dT). Neither DMXAA nor

**FIGURE 3.** STING binds to DMXAA and is a direct sensor for DMXAA. (A) 293T cells were transfected with empty vector or mSTING, and cell lysates were UV–cross-linked to c-di-GMP in the presence of cold competing c-di-GMP or DMXAA in 10-fold serial dilutions. (B) His6-mSTING 138–378 (1 μg) was analyzed as described in (C). (C) Left panel, Thermal shift analysis of mouse STING in the presence of GMP, c-di-GMP, c-di-AMP, and DMXAA. The melting temperature shifts in the presence of the ligands are plotted in the graph to the right of the melting curves. Data are representative of two independent experiments (A–C).
c-di-AMP turned on expression of this panel of immune genes in human cells (Fig. 4B).

On the basis of these findings, we hypothesized that the differential responses of human and mouse cells to DMXAA might be due to different capacities of human and mouse STING to respond to DMXAA. To test this possibility directly, we compared the ability of human and murine STING to reconstitute DMXAA signaling in 293T cells. All of the data presented thus far used murine STING in the reconstitution assays and purified murine STING–CTD in the binding assays. Unlike murine STING, expression of human STING in 293T cells failed to render these cells responsive to DMXAA (Fig. 4C). This was true not only for DMXAA but also for cyclic-di-GMP (Fig. 4C, right panel) and cyclic-di-AMP (data not shown). The inability of human STING to restore DMXAA signaling was also observed when an ISG54–ISRE reporter or an NF-kB luciferase reporter (Fig. 4D) were used. *p < 0.05 for the comparison of DMXAA or c-di-GMP relative to vector control. 293T cells were transfected as above and stimulated with DMXAA and cyclic-di-GMP. Endogenous TBK1 was immunoprecipitated and analyzed as in Fig. 1. Data are presented as the mean ± SEM of one experiment representative of three experiments (A), (B–E) Data are representative of three separate experiments.

Our binding data indicated that the CTD of STING was important in binding to both DMXAA and c-di-nucleotides, therefore, we next generated chimeric STING molecules in which the CTD of human STING was replaced with that of the mouse and vice versa and examined the ability of these chimeric STING molecules to signal in response to DMXAA and c-di-GMP. We transfected these constructs into 293T cells and found that all were expressed to a similar degree (Fig. 5A). Although murine STING conferred DMXAA and c-di-GMP responsiveness to 293T cells, as we had previously shown, expression of human STING or a chimeric mouse-human STING (in which the CTD of the mouse was replaced with that of humans) failed to facilitate DMXAA-induced IFN-β PRDIII-I reporter gene activation (Fig. 5B). In contrast, when the CTD of human STING was replaced with that of mouse STING, this h-m-STING chimera responded to both DMXAA and c-di-GMP. We also monitored TBK1 activation as well as phosphorylation of IRF3 in 293T cells expressing human STING.
STING, mouse STING, and the STING chimeras. Only the mouse STING and the h-m-STING chimeric molecules facilitated DMXAA-induced TBK1 and IRF3 activation (Fig. 5C).

We also conducted similar experiments in cells stimulated with cyclic-di-GMP and found that only mouse STING reconstituted responses to cyclic-di-GMP (Fig. 5D). In light of these data we next examined the binding of DMXAA to purified human STING CTD using the thermal shift assay described above. No significant enhancement of Tm was observed when the human STING CTD was incubated with DMXAA (Fig. 5E). In contrast, c-di-GMP and c-di-AMP both bound human STING, consistent with previous structural studies and the strict sequence conservations for the c-di-GMP-binding residues between human and mouse STING (Supplemental Fig. 2, sequence alignment, residues highlighted in...
red). Collectively, these data indicate that mouse STING binds both cyclic-di-nucleotides and DMXAA and signals TBK1–IRF3 activation as well as NF-κB activation. In contrast, human STING fails to bind DMXAA and activate TBK1. Although human STING still binds cyclic-di-nucleotides, this binding appears to be insufficient to trigger downstream TBK1–IRF3 activation. Taken together, these data demonstrate that unlike mouse STING, human STING lacks the ability to respond to DMXAA and cyclic dinucleotides.

Discussion
Vascular disrupting agents disrupt blood flow leading to the rapid and irreversible collapse of the established tumor vasculature. The acute ischemia that results leads to widespread tumor necrosis and a significant reduction in tumor burden. Flavone derivatives represent a novel class of vascular disrupting agents and include FAA and DMXAA. FAA was discovered based on its unexpectedly potent antitumor activity in mice (42). The related compound DMXAA was found to be much more potent in mouse cancer models, and despite showing great promise in early phase I and II trials ultimately failed in phase III clinical trials (43, 44). DMXAA kills tumors through complex mechanisms leading to the induction of tumor hemorrhagic necrosis. DMXAA treatment also activates tumor-associated macrophages (TAMs) to increase local and systemic levels of inflammatory cytokines such as TNF-α, which are also important in antitumor activity of this drug (3, 45, 46).

Considerable efforts have focused on the molecular mechanism of action of DMXAA. Early studies provided insights into similarities and differences in gene induction in murine macrophages stimulated by DMXAA when compared with Escherichia coli LPS (7, 46, 47). Compared with LPS, DMXAA potently induced a more limited subset of genes that included IFN-β and IP-10, but relatively low levels of TNF-α and other proinflammatory cytokine genes (7). Much like TNF-α, the IFNs have been known for decades to possess pleiotropic effects on tumor growth. The antitumor properties of IFNs are well known (48) and a number of IFNs are currently used in the clinic to treat more than a dozen malignancies. Notably, type I IFNs represent the first human recombiant proteins to be used successfully in the clinic to treat cancer (49). Although TAMs have been shown to promote the growth of tumors by promoting a local cytokine milieu that tends to favor tumor growth (50), in response to DMXAA, TAMs may engender a CD8+ T cell–mediated attack on the tumor (5). Type I IFNs may play a direct role in mediating these effects (51, 52). In addition to the direct effects of IFN on the protection of the host from cancer, these cytokines also have numerous indirect effects resulting from downstream autocrine and paracrine effects. Type I IFN has also been shown in numerous studies to have antiangiogenic functions (53, 54), whereas IFN-inducible genes, such as the CXC chemokine IP-10, have angiostatic properties (55). Considerable evidence is also coming forth that type I IFNs and other proinflammatory cytokines, and other regulators of inflammation. In all cases, induction of these genes was abrogated in macrophages lacking STING suggesting that STING mediates both the IRF3 and NF-κB signaling pathways. It is important to point out that although DMXAA can turn on NF-κB target genes such as IL-1α and IL-6, the induction of these genes is very modest by comparison with IFN and ISG induction. STING was first identified as an endoplasmic reticulum–residing protein relaying signals to IRF activation and IFN transcription from a variety of stimuli, especially cytosolic dsDNAs (30–32). STING-deficient cells fail to produce type I IFN in response to transfection with dsDNA or infection with HSV-1 (30). STING was also shown to be essential for IFN production in response to c-di-nucleotides such as c-di-GMP and c-di-AMP (56). A mutant mouse strain, Goldenticket, which harbors a missense mutation (I199N) in the STING protein, fails to produce detectable protein and to activate IFN production in response to c-di-GMP (56). Both c-di-GMP and c-di-AMP are second messengers secreted by bacteria (56). Surprisingly, STING was shown to function as a direct sensor for c-di-GMP, and this interaction mapped to the cytosolic C terminal domain and was shown to be critical for cellular responses to c-di-nucleotides (33).

STING contains four transmembrane helices and a globular carboxy-terminal domain (CTD), predicted to be localized in the cytosol. The cytosolic domain does not exhibit significant sequence homology to any proteins with known structures. Within the cytosolic domain, a recent study showed that the very C-terminal tail (CTT) interacts with and activates TBK1 and IRF3 in vitro (57). The CTT of STING is also important for maintaining the autoinhibited state of inactive STING (58). Structural studies suggest that the CTT is involved in stable STING–dimerization and that c-di-nucleotides bind to the CTD/groove between two STING homomers (37–41, 58) It is suspected that the binding of c-di-nucleotides would relieve this autoinhibitory state, expose the CTT, and stabilize STING dimers with c-di-nucleotides buried in the cleft between the interacting protomers (39, 41, 58) (shown in Supplemental Fig. 3).

In this study, we found that DMXAA behaves similarly to c-di-nucleotides and binds directly to the mouse STING CTD. Our 293T reconstitution studies, combined with our binding studies, indicate that mouse STING serves as a direct sensor for this chemotherapeutic drug. Unlike mouse STING, NOD2 and DDX41 both failed to reconstitute signaling to DMXAA. STING binds directly to DMXAA and the competition experiments indicate that DMXAA competes with cyclic-di-GMP for binding. Surprisingly, we found that only murine STING could serve as a direct sensor for DMXAA. Comparison of the ability of mouse and human STING to reconstitute DMXAA signaling in 293T cells revealed a clear difference between mouse and human STING in terms of DMXAA signaling. The failure of DMXAA to induce STING-dependent signaling in humans was mapped to the CTT, because a chimeric murine STING molecule with the CTD from humans was unresponsive to DMXAA, whereas a chimeric human STING molecule with the CTD from mouse was fully competent for signaling in response to DMXAA. Binding studies indicate that human STING did not bind to DMXAA, suggesting that nonconserved residues between hSTING and mSTING are part of the DMXAA binding surface. It is possible that DMXAA and c-di-GMP have overlapping but nonidentical binding surfaces on mouse STING, and one such model of the STING CTD in complex with DMXAA is shown in Supplemental Fig. 4. Another possibility is that DMXAA may bind near the mSTING dimer interface residues D273 or K275, which are not conserved in hSTING (see Supplemental Figs. 2, 3). Such binding may change the mSTING dimer interface and modulate STING autoinhibition. STING is autoinhibited by an intramolecular interaction between the cyclic-di-GMP–binding domain and the CTT and that c-di-GMP releases STING from this autoinhibition by displacing the CTT near the dimer interface. If such an autoinhibitory model involving the CTT is correct (58), then DMXAA or c-di-GMP...
may displace the CTT from a mSTING autoinhibited state to induce signaling, but they do not necessarily need to bind at the same site.

It is tempting to speculate that the failure of DMXAA in humans might relate to its inability to engage the STING pathway in human cells. Although murine STING serves as a sensor for DMXAA, activating a robust IFN and inflammatory response important for the antitumor effect, a defect in this capability could drastically affect the therapeutic efficacy of DMXAA in humans. The inability of DMXAA to bind to human STING and activate STING signaling in humans could, therefore, provide important insights that may help explain the failure of DMXAA in clinical trials for human cancer. The inability of cyclic di-nucleotides to trigger human STING signaling was also surprising. Previously, a number of mouse STING mutants were reported to retain their ability to associate with c-di-GMP but failed to induce IFN production (33). Several such mutants that “decouple” c-di-GMP binding and IFN induction are at the nonconserved residues between human and mouse STING (see sequence alignment Supplemental Fig. 2, highlighted in yellow). Conceivably, these and perhaps other nonconserved residues may play a role in the species-specific immune responses to c-di-GMP. The inability of these molecules to engage human STING may lessen enthusiasm for the development of these immunostimulatory molecules as immunotherapeutics or vaccine adjuvants for clinical use. In addition, these findings might also pave the way to redirect efforts to identify and develop analogs of DMXAA that could bind to human STING and signal and therefore have efficacy in humans.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure Legends.

**Supplemental Figure 1.** STING but neither NOD2 nor DDX41 confers responsiveness of 293T cells to DMXAA. 293T cells were transfected with either empty vector, mSTING, mNOD2, hNOD2, DDX41 or MyD88 as indicated in the presence of either an IFN-β (A) or NFκB (B) luciferase reporter gene. Transfected cells were stimulated with DMXAA at the doses indicated for 18 hours and luciferase activity was measured. Data are presented as the mean ± s.e.m of one experiment representative of three experiments.

**Supplemental Figure 2 Sequence alignment of the human and mouse STING.** Identical residues are marked with “*”, conserved residues with “:”, and partially conserved with “.”. Residues involved in c-di-GMP binding in hSTING and their mSTING counterparts are in red, those at the dimerization interface in green, those shown to be essential for signaling but not for c-di-GMP binding in yellow.

**Supplemental Fig 3. Ribbon diagram of the human STING CTD structure.** The two STING CTDs are colored green and cyan, respectively. The bound c-di-GMP is shown in sticks (orange), and residues different between human and mouse STING are shown in space-filling spheres, which are distal to the c-di-GMP binding pocket.

**Supplemental Fig 4. Ribbon diagram of a hypothetical model of the STING CTD in complex with DMXAA.** The two STING CTDs are colored green and cyan, respectively. The bound DMXAA molecules are shown in sticks (orange).
Suppl. Figure 1

A. IFNβ luciferase (fold induction)

B. NFkB-luciferase (fold induction)

C. IFNβ luciferase (fold induction)
Suppl. Fig. 3
Suppl. Fig. 4.