TBK1 recruitment to STING activates both IRF3 and NF-κB that mediate immune defense against tumors and viral infections

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The induction of type I interferons through the transcription factor interferon regulatory factor 3 (IRF3) is considered a major outcome of stimulator of interferon genes (STING) activation that drives immune responses against DNA viruses and tumors. However, STING activation can also trigger other downstream pathways such as nuclear factor κB (NF-κB) signaling and autophagy, and the roles of interferon (IFN)-independent functions of STING in infectious diseases or cancer are not well understood. Here, we generated a STING mouse strain with a mutation (S365A) that disrupts IRF3 binding and therefore type I interferon induction but not NF-κB activation or autophagy induction. We also generated STING mice with mutations that disrupt the recruitment of TANK-binding kinase 1 (TBK1), which is important for both IRF3 and NF-κB activation but not autophagy induction (L373A or ΔCTT, which lacks the C-terminal tail). The STING-S365A mutant mice, but not L373A or ΔCTT mice, were still resistant to herpes simplex virus 1 (HSV-1) infections and mounted an antitumor response after cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) treatment despite the absence of STING-induced interferons. These results demonstrate that STING can function independently of type I interferons and autophagy, and that TBK1 recruitment to STING is essential for antiviral and antitumor immunity.

STING trafficking induces noncanonical autophagy to clear DNA or pathogens from the cytosol (6). The role of STING-induced type I IFNs has been extensively studied in infectious diseases and cancer, but the IFN-independent functions of STING in diseases are poorly understood. The core components of the cGAS-STING pathway are conserved from bacteria to humans (14–16), but only vertebrate STING contains the CTT that binds to TBK1 and IRF3 to induce IFNs. Nonvertebrate STING functions against pathogens without inducing type I IFNs: *Nematostella vectensis* STING induces autophagy (6) while *Drosophila melanogaster* STING induces autophagy and NF-κB activation (17, 18) to restrict viral infections.

Mice lacking cGAS or STING are highly susceptible to acute herpes simplex encephalitis (HSE) caused by herpes simplex virus (HSV) infection (19–21). Type I IFN signaling was suggested as the major mechanism of this antiviral effect as mice deficient in the interferon-α/β receptor (IFNAR) failed to clear viruses (22). However, the Toll-like receptor 3 (TLR3) pathway also induces IFNs in response to HSV-2 (23), and the direct role of STING-induced type I IFNs in antiviral responses in vivo has not been studied. In vitro, STING-induced autophagy was shown to be important for initially clearing HSV-1 from infected cells (6), suggesting that STING may trigger type I IFN-independent antiviral responses in addition to inducing IFN production.

The cGAS-STING pathway is important for immune defense against infection and cancer. STING activation triggers multiple signaling cascades leading to activation of IRF3, NF-κB, and autophagy. By generating mice harboring mutations of STING that specifically inactivate different signaling cascades, we found that ablation of IRF3 activation, which is essential for the induction of type I interferons, was not sufficient to abolish the immune defense against virus infection and cancer in mouse models. Rather, impairing the ability of STING to recruit TBK1, which is important for activating both IRF3 and NF-κB, abolished the immune defense functions of STING. These results demonstrate that the recruitment of TBK1 to STING has functions that are broader than activating IRF3 and inducing type I interferons.

Significance

The role of STING-induced type I IFNs has been extensively studied in infectious diseases and cancer, but the IFN-independent functions of STING in diseases are poorly understood. The core components of the cGAS-STING pathway are conserved from bacteria to humans (14–16), but only vertebrate STING contains the CTT that binds to TBK1 and IRF3 to induce IFNs. Nonvertebrate STING functions against pathogens without inducing type I IFNs: *Nematostella vectensis* STING induces autophagy (6) while *Drosophila melanogaster* STING induces autophagy and NF-κB activation (17, 18) to restrict viral infections.

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Tumor-derived DNA that accumulates in immune cells also activates the cGAS-STING pathway to promote cancer immune surveillance; STING was necessary to induce tumor-specific T cells and to control tumor growth (24). Activating cGAS or STING augmented antitumor immunity, providing a therapeutic effect in various tumor models (25). This antitumor effect was reduced in the absence of type I IFN signaling (26, 27), suggesting a critical role for IFNs in the STING-induced antitumor effect. However, the antitumor effects of other STING functions have not been well-characterized. During radiation-induced STING activation, the canonical NF-xB pathway enhanced the antitumor effect by promoting type I IFN expression whereas the noncanonical NF-xB pathway impeded the antitumor effect by reducing type I IFN expression (28). In addition to regulating IFN expression, NF-xB signaling not only induces inflammatory cytokines that promote immune cell functions but also promotes an inflammatory tumor microenvironment that can promote tumor survival and metastasis (29).

In order to study the type I IFN-independent functions of STING in diseases, we generated three different STING mutant mouse strains that exhibited partial loss of STING functions: S365A, L373A, and C-terminal tail truncation (ΔCTT). Using these mouse models, we observed that STING-S365A mutant mice, which lack STING-induced IRF3 activation but retain NF-xB activation, were still resistant to HSV-1 infection and mount an antitumor response against the Lewis lung cancer model after cGAMP treatment. This antiviral and antitumor effect was not found in the L373A and ΔCTT mutants that were defective in recruiting TBK1 and therefore unable to activate IRF3 and NF-xB. Activation of the STING-S365A mutant stimulated immune cells and instigated NF-xB–induced immunostimulatory gene expression. Our study demonstrates the critical role of TBK1 recruitment by STING in antiviral and antitumor immunity and reveals a type I IFN-independent function of STING that is critical for understanding STING-related diseases. This result is in line with recent studies reporting the type I IFN-independent antiviral effect in STING-S365A mice (30, 31) and further defines the mechanism using STING-L373A mice.

Results

STING Mutant Mice with Distinct Signaling Defects. In order to determine the role of STING activation in different disease models, we generated three STING mutant mice using the CRISPR-Cas9 system (SI Appendix, Table S1). The S365A mutation is defective in phosphorylation, thereby disrupting recruitment of IRF3 while retaining the ability to recruit TBK1 (10). The L373A mutation disrupts the TBK1 recruitment motif and is defective in both IRF3 and NF-xB activation (8). The ΔCTT mutation has a stop codon after amino acid 339, thereby producing a truncated protein lacking the C-terminal tail (residues 340 to 378) that is important for TBK1 binding. All three mutants are expected to be capable of binding cGAMP and inducing autophagy (6). We isolated bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophages (BMDMs) from the STING mutant mice and stimulated the cells with the mouse STING agonist 5,6-dimethylxanthone-4-acetic acid (DMXAA), which is more permeable to cells than cGAMP. Western blotting of the protein lysates from BMDCs (Fig. 1D) and BMDMs (SI Appendix, Fig. S1A) showed gradual loss of the downstream signaling pathways. All BMDMs expressed STING or its mutant proteins except the STING-Goldenticket (StingΔ) mutant, which carries an I199N null mutation that renders the loss of STING expression (32). Notably, STING-S365A, but not STING-L373A, preserved the mobility shift of STING in the Western blot, indicating TBK1-mediated phosphorylation of other residues at the CTT as previously described (9, 10). All mutants lacked phosphorylation of the STING-S365 residue, IRF3, and STAT1 upon stimulation, indicating defects in IRF3 activation and type I IFN signaling (9) (Fig. 1A and SI Appendix, Fig. S1A). Consistently, all mutants were deficient in IRF3 nuclear translocation (Fig. 1B and SI Appendix, Fig. S1B) and did not produce IFNβ after DMXAA treatment (Fig. 1C). Despite the lack of IFN signaling, STING-S365A cells retained normal activation of TBK1 and IKKe, as shown by their phosphorylation. Phosphorylation of p65 and IκBα, indicating NF-xB signaling, was intact in the STING-S365A cells (Fig. 1A and SI Appendix, Fig. S1A). DMXAA-treated STING-L373A and ΔCTT mice lacked IFNβ and NF-xB signaling and were protected by the absence of phosphorylated STAT1, TBK1, IKKe, p65, and IκBα (Fig. 1A and SI Appendix, Fig. S1A). Consistent with the Western blotting data, p65 nuclear translocation was deficient in L373A and ΔCTT cells but not in S365A cells (Fig. 1B and SI Appendix, Fig. S1B). Autophagy induction, which is indicated by the conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3) from LC3-I (upper band in longer exposure blot) to LC3-II (lower and darker band in normal and longer exposure blots), was intact in S365A and L373A cells (Fig. 1A). This is consistent with a previous study showing that deletion of the CTT from STING did not impair LC3 lipidation (6). However, the autophagy induction seems to be weaker in the STING-ΔCTT cells (Fig. 1A), which might be due to a lower level of expression in the STING-ΔCTT protein in BMDCs.

STING-S365A Mice, but Not L373A or ΔCTT Mice, Are Resistant to HSV-1 Infection. STING-deficient mice are susceptible to HSV-1 infection whereas wild-type (WT) mice survive and clear the virus within a few days (20). To determine which functions of STING led to this protection against HSV-1, we retroorbitally infected WT, Ifnar1−/−, and different STING mutant mice and assayed their responses. Ifnar1−/− mice succumbed to the virus infection early on as previously reported (Fig. 2A and SI Appendix, Fig. S2A) (22). Production of serum IFNβ was confirmed 6 h after infection in WT and Ifnar1−/− mice but not in any of the STING mutant mice (Fig. 2B). However, lower levels of IFNβ were detected in the sera of WT and all STING mutant mice 2 d after infection, indicating that pathways other than STING can detect HSV-1 and produce IFNβ at a later time point (SI Appendix, Fig. S2B). Despite the lack of STING-induced IFNβ, STING-S365A mice were partially resistant to HSV-1 infection as indicated by their survival rate, body weight change, and viral titer in the brain being similar to those measurements from WT mice (Fig. 2A and C and SI Appendix, Fig. S2A). In contrast, the mice harboring the L373A mutation and C-terminal deletion (ΔCTT) of STING all succumbed to HSV-1 infection and had high viral titers in the brain, similar to the STING-deficient mice (StingΔ) (22). These results indicate that TBK1 recruitment to STING, which is important for both IRF3 and NF-xB activation, is essential for immune defense against HSV-1. Because the STING-L373A mutant, and to a lesser extent the ΔCTT mutant, can still induce autophagy, the results also suggest that autophagy induction by STING alone is insufficient to protect the mice from HSV-1 infection in vivo.

All BMDCs harboring the STING mutations secreted markedly less (~1,000-fold reduction) but still detectable levels of IFNβ after HSV-1 infection (SI Appendix, Fig. S2C), indicating that STING is the main pathway producing IFNβ after initial infection although other pathways may still partially contribute. Furthermore, STING-induced type I IFN provided an immediate and direct antiviral effect in vitro; all IFN-deficient mutant BMDCs showed more green fluorescent protein-positive (GFP+) cells after HSV-GFP infection (SI Appendix, Fig. S2D) and acquired higher viral genome equivalent (VGE) counts after HSV-1 infection (SI Appendix, Fig. S2E). Despite this antiviral effect in vitro, STING–induced IFN was dispensable for protecting the STING-S365A mice from HSV-1 infection (Fig. 2A).
**STING Activation Promotes a Type I IFN-Independent Antitumor Effect.** The cGAS-STING pathway is an endogenous pathway that detects tumors and initiates immune responses (24); thus, host cGAS was required for the antitumor effect of the immune checkpoint inhibitor anti–PD-L1 in the B16 melanoma mouse model (33). Here, we used the Lewis lung carcinoma (LL2) tumor model to study the antitumor effect of STING activation in immunologically cold tumors. In order to confirm the spontaneous detection of the LL2 tumor by the immune system, we implanted LL2 tumors into IFNβ-luciferase reporter mice. IFNβ-luciferase reporter mice showed weak basal levels of luciferase signal without tumor implantation (SI Appendix, Fig. S3A), as was observed previously (34). However, a stronger IFNβ-driven luciferase signal was found at the tumor area in a cGAS-dependent manner (SI Appendix, Fig. S3A and B), indicating the detection of the LL2 tumor by cGAS. As previously reported (35), the LL2 tumor was resistant to anti–PD-L1 therapy (SI Appendix, Fig. S3C) despite the induction of PD-L1 by IFNγ or IFNβ treatment (SI Appendix, Fig. S3D) and the expression of PD-L1 on the cell surface of tumor cells isolated from mice (SI Appendix, Fig. S3E and F).

Despite the poor immunogenicity of the LL2 tumors, intratumoral cGAMP treatment reduced the LL2 tumor growth in a dose-dependent manner (Fig. 3A). Sting<sup>−/−</sup> mice completely lost this antitumor effect, indicating that the activation of host STING is essential for the antitumor effect of cGAMP (SI Appendix, Fig. S4A). LL2 tumor cells also have a functional STING pathway that induces TBK1 phosphorylation and CXCL10 expression upon activation (SI Appendix, Fig. S4C and D). To determine the role of tumor STING in this cGAMP-induced antitumor response, we generated an LL2-STING<sup>−/−</sup> clonal cell line using CRISPR-Cas9 that abrogated STING downstream signaling (SI Appendix, Fig. S4C and D). Consistent with this result, LL2-STING<sup>−/−</sup> cells implanted into Sting<sup>−/−</sup> mice also abrogated the residual interferon-stimulated gene (ISG) levels in the tumor after intratumoral cGAMP treatment (SI Appendix, Fig. S4E). WT mice implanted with this LL2-STING<sup>−/−</sup> cell line still showed a significant reduction in tumor size after cGAMP treatment while inducing ISGs in the tumor and draining lymph node, indicating that host STING is sufficient to mediate the antitumor effect of cGAMP (SI Appendix, Fig. S4B and E).

We also treated LL2 tumors with cGAMP at later time points (days 9 and 13) and analyzed the tumor-infiltrating immune cells (SI Appendix, Fig. S5A–E). The tumor weights were comparable 1 d after the last cGAMP treatment (SI Appendix, Fig. S5A), but the cGAMP-treated group showed more recruitment of CD45<sup>+</sup>...
cells into the tumor (SI Appendix, Fig. S5B). The percentage of CD8$^+$ T cells and natural killer (NK) cells did not increase, and the percentage of CD4$^+$ T cells decreased after cGAMP treatment (SI Appendix, Fig. S5C); however, all tumor-infiltrating lymphocytes up-regulated CD69, indicating their activation (SI Appendix, Fig. S5D). The most drastic change induced by cGAMP treatment in the tumor cell population was in cells from the myeloid lineage; the percentage of neutrophil-like cells increased while monocyte-like cells decreased (SI Appendix, Fig. S5E). cGAMP treatment, along with inducing IFN$\gamma$, CXCL10, and IFN$\gamma$2, also up-regulated nitric oxide synthase 2 (NOS2) and down-regulated arginase 1 (ARG1) in the tumor, which suggests polarization toward the N1 phenotype (SI Appendix, Fig. S5F). We then depleted CD8$^+$ T cells and NK cells in the tumor-implanted mice and confirmed the depletion in the spleen until end point (SI Appendix, Fig. S5G). Depleting NK cells (SI Appendix, Fig. S5H), but not CD8$^+$ T cells (SI Appendix, Fig. S5I), partially reduced the antitumor effect of cGAMP.

Type I IFN signaling is essential for the therapeutic effect of cGAMP in various tumor models (26, 27). For the LL2 tumor model, cGAMP treatment showed a weaker but still significant reduction in tumor volume for Ifnar1$^{-/-}$ mice (Fig. 3B). As type I IFN may act on the LL2 cells, we injected anti–IFNAR-1 antibodies into Ifnar1$^{-/-}$ mice tumors to further block type I IFN signaling. cGAMP still exerted a significant antitumor effect even with this treatment (SI Appendix, Fig. S6A), which further reduced the ISG levels in the tumor and spleen (SI Appendix, Fig. S6B). These results demonstrate that cGAMP exerts both type I IFN-dependent and -independent antitumor effects.

Next, we tested the effect of cGAMP treatment on LL2 tumors in STING mutant mice to study the role of the downstream signaling pathways. cGAMP treatment reduced tumor sizes only in WT and STING-S365A mice but not in L373A or ΔCTT mice (Fig. 3C), even though all STING mutant mice lacked serum IFN$\beta$ production in response to intratumoral cGAMP treatment (Fig. 3D). Our data demonstrate that intratumoral cGAMP treatment exerts a therapeutic effect on LL2 tumors by activating host STING, which triggers both type I IFN-dependent and type I IFN-independent antitumor effects. Autophagy is not sufficient to drive antitumor immunity as STING-L373A mice lost the antitumor response. STING-S365A mice retained NF-kB activation, which may provide an immunostimulatory effect and compensate for the loss of IFN signaling.

Transcriptome Analysis of STING-Induced Type I IFN-Independent Genes. In order to identify the IFN-independent functions of STING that mediate the antiviral and antitumor effect, we performed a transcriptome analysis of STING mutant BMDMs stimulated with DMXAA. WT cells induced a large number of genes including IFN$\beta$, ISGs, and other cytokines (SI Appendix, Fig. S7A). Some of these genes were expressed at lower levels in STING-S365A mutant cells but not in L373A or ΔCTT, or STING$^B$ cells. Interestingly, DMXAA-treated S365A BMDMs also induced expression of several genes at levels higher than even WT cells did (SI Appendix, Fig. S7B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the transcriptome data shows the activation of cytokine signaling pathways in S365A mutant cells; specifically, genes in the NF-kB signaling pathway were more enriched in the S365A cells than in the WT cells (SI Appendix, Fig. S7B). The top 20 up-regulated genes in the S365A cells included NF-kB–induced genes such as Cxcl1, Cxcl2, Tnf sf9 (4-1BBL), and Ptg2 (COX2) (Fig. 4A). qRT-PCR analysis of stimulated BMDMs and BMDMs confirmed the expression of these genes (Fig. 4B–D and SI Appendix, Fig. S8). None of the STING mutant cells induced IFN$\alpha$, IFN$\beta$, or ISGs (Fig. 4B and C and SI Appendix, Fig. S8A); STING-S365A cells, however, expressed CXCL1, CXCL2, 4-1BBL, and COX2 at higher levels than even WT cells did (Fig. 4D and SI Appendix, Fig. S8B). It is possible that these NF-kB–regulated genes are more highly expressed in the absence of activated IRF3.

In cGAMP-treated tumors, IFN$\beta$ production was the highest 4 h after a cGAMP injection as shown by the tumor luciferase activity in the IFN$\beta$-luciferase reporter mice (Fig. 4A, Fig. S9A). At this time point, cGAMP-treated WT mice, but not any STING mutant mice, expressed ISGs and IFN$\beta$ in the spleen (SI Appendix, Fig. S9B). As tumor STING is functional, tumors from STING mutant mice still induced low levels of IFN$\beta$ and ISGs after cGAMP treatment; this expression level, however, was significantly lower than those in the tumors of the WT mice (SI Appendix, Fig. S9C). Tumors from WT and S365A mutant mice, but not from other mutants, expressed NF-kB–induced genes such as Cxcl1 and Cxcl2 after cGAMP treatment. Tumor necrosis factor $\alpha$ (TNF$\alpha$) and interleukin-1$\beta$ (IL-1$\beta$), which are regulated by both IRF3 and NF-kB, were down-regulated in the tumors of all STING mutant mice (SI Appendix, Fig. S9C), likely due to the lack of IRF3 activation. These up-regulated NF-kB–induced genes may play a role in activating innate and adaptive immune cells, providing an immunostimulatory effect for antitumor immunity.

Type I IFN-Independent Functions of STING Have Immunostimulatory Effects. To investigate whether the type I IFN-independent functions of STING have immunostimulatory effects, we analyzed the activation markers of STING mutant immune cells upon stimulation. WT and STING-S365A BMDMs, but not BMDMs from other STING mutants, up-regulated major histocompatibility complex class II (MHC-II) and the costimulatory molecules CD86, CD80, and CD40 after DMXAA treatment (Fig. 5A). Similar to WT cells,
STING-S365A splenocytes stimulated with DMXAA up-regulated CD69 on CD4+ and CD8+ T cells, B cells, and NK cells, although the CD69 expression level was lower in CD4+ T cells from S365A mice than in those from WT mice (Fig. 5B). In contrast, DMXAA-induced up-regulation of CD69 was abolished in all cells from L373A, ΔC TT, and StingΔ mice (Fig. 5B).

We then immunized the STING mutant mice with the model antigen ovalbumin (OVA) with cGAMP. After prime-boost immunization, cGAMP-treated WT mice displayed higher levels of serum anti-OVA immunoglobulin G (IgG) compared with the WT mice treated only with OVA (Fig. 5C). This adjuvant effect of cGAMP was retained in STING-S365A mice, albeit at a lower level, and was abrogated in L373A, ΔC TT, and StingΔ mice (Fig. 5C). However, OVA and cGAMP immunization did not increase OVA-specific CD8+ T cell populations (Fig. 5D). T cell activation by IFN-independent functions of STING in vitro (Fig. 5B), but not in vivo (Fig. 5D), suggests that stronger stimulation or activation of T cell STING may be needed for T cell activation in vivo. Altogether, these data demonstrate that STING can activate innate and adaptive immune cells in vitro and elicit significant antibody responses in a manner that depends on TBK1 recruitment but not type I IFNs.

Discussion

Prior studies on antiviral and antitumor immunity revealed that the cGAS-STING pathway is critical for the recognition of DNA viruses and tumors and initiation of the subsequent immune response. Although STING activation leads to multiple outcomes such as proinflammatory cytokine production and autophagy, most studies on STING-related diseases have focused on type I IFN production as the major downstream player. In order to dissect the role of distinct STING signaling functions in vivo, we generated three different STING mutant mice: S365A, L373A, and ΔC TT. Here, we show that STING-S365A mutant mice that lost type I IFN induction are still resistant to HSV-1 infection and can still mount an antitumor response after cGAMP treatment, suggesting an IFN-independent antiviral and antitumor effect mediated by STING. Such an effect was not observed in STING-L373A that failed to recruit TBK1, indicating STING mediates antiviral and antitumor functions through a mechanism that depends on TBK1 recruitment but is independent of type I IFN induction. Since the STING-L373A mutant induces autophagy normally, the results also suggest that STING-induced autophagy is not sufficient to drive an antiviral or antitumor response in vivo.

Two recent papers reported that STING-S365A mice were resistant to HSV-1 infection (30, 31), similar to our results. In one of the studies, it was suggested that STING-induced, TBK1-dependent autophagy explains this effect as TBK1 was required for the antiviral effect and STING-ΔC TT mice, which lost autophagy induction, were susceptible to HSV-1 infection (31). However, our study provides another STING mutant model, L373A, that lost TBK1/Ikkβ: activation but retained autophagy induction. We showed that STING-L373A mice were susceptible to HSV-1 infection, indicating that autophagy alone is not sufficient to drive the antiviral response by STING in vivo (Fig. 2D). Our data confirm that TBK1 is dispensable for STING-induced LC3 lipidation in primary cells (Fig. 1A), which was previously only described in mammalian cell lines (6, 7). However, our result does not determine if STING-induced autophagy is essential for restricting HSV-1 in vivo; such analysis would require a STING autophagy-specific mutation, which has yet to be discovered. Nevertheless, inflammation and immune cells activated by TBK1 recruitment to STING may play a more critical role in vivo to restrict viral infections. The NF-κB pathway is a strong candidate for the IFN-independent STING-induced antiviral responses. We showed that NF-κB–driven genes such as CXCL1, CXCL2, and 4-1BBL are up-regulated in STING-S365A cells that may contribute to viral resistance (Fig. 4). Monocytes and neutrophils were previously reported to restrict the replication and spread of HSV (37–39); CXCL1 and CXCL2 may exert an antiviral function by recruiting monocytes and neutrophils to the infected site. 4-1BBL was shown to expand lymphocytic choriomeningitis...
virus-specific CD8+ T cells and thus may contribute to T cell-mediated antiviral immunity (40). Further studies are needed to determine whether the STING-induced NF-κB signaling or other pathways elicited by STING play a major role in defending against pathogen infections in the absence of STING-induced type I IFNs. The specific role of type I IFNs in the antiviral response also requires further study. STING mutant mice that lack STING-induced type I IFN production survived HSV-1 infection (Fig. 2A) (30, 31) while IFNAR-deficient mice were highly susceptible to HSV-1 infection (22). These results suggest that STING-independent IFN production in response to HSV-1 infection is necessary for the antiviral response (41). TLR2, TLR3, and TLR9 have been reported to detect HSV-1 and induce type I IFNs (42–44). In the absence of STING-induced IFNs, these pathways may compensate for defective STING signaling and play a vital role in antiviral immunity. Indeed, we observed comparable levels of serum IFNβ in WT and all STING mutant mice 2 d postinfection (SI Appendix, Fig. S2B). Which pathway produces this type I IFN and how this later IFN production provides an antiviral effect need to be investigated further. Nevertheless, the cGAS-STING pathway is responsible for the rapid initial IFN response after infection (Fig. 2B). Moreover, since the STING-L373A mutant mice are completely susceptible to HSV-1 infection, the IFN-independent immune defense functions of STING cannot be compensated by another pathway.

Our study revealed that STING activation provides a potent antitumor effect against LL2 through both a type I IFN-dependent and -independent mechanism. Based on the significant antitumor effect by cGAMP in STING-S365A mice, but not in -L373A mice, the NF-κB pathway is suggested as the main candidate for providing this antitumor effect. NF-κB signaling and the subsequent inflammation are known to induce both antitumor and protumor responses. Chronic and persistent low-level inflammation recruits suppressive immune cells such as regulatory T cells and myeloid-derived suppressor cells, which inhibit antitumor immune responses; NF-κB signaling also promotes metastasis by inducing cancer cell proliferation and angiogenesis (45). Chronic activation of STING by tumors was also implicated in promoting tumor metastasis (29, 46). However, acute activation of the NF-κB pathway promotes perforin and IFNγ expression by NK cells (47, 48). In addition, NF-κB in CD4+ T cells was essential to reject fibrosarcoma tumors and to activate tumor-specific T cells (49). We observed activation of innate and adaptive immune cells by STING in an IFN-independent manner in vitro (Fig. 5B). In the LL2 tumor model, the antitumor effect of cGAMP was partially dependent on NK cells (SI Appendix, Fig. S5B). In this regard, interaction of 4-1BB on NK cells with 4-1BBL–expressing cells promoted NK cell proliferation, and the agonistic anti–4-1BB has shown an NK cell-dependent antitumor effect on mastocytoma (50, 51). cGAMP treatment...
recruited Ly6G+ neutrophil-like cells into the tumor (SI Appendix, Fig. S5E) and induced NOS2 (N1 phenotype) while down-regulating ARG1 (N2 phenotype) in the tumor (SI Appendix, Fig. S5F). N1 neutrophils have the potential to direct tumor cell death through the production of reactive oxygen species (36). Mechanistically, reactive oxygen species acted on the H2O2-dependent calcium channel TRPM2 on cancer cells, including LL2 cells, to mediate lethal influx of calcium ions (52). Moreover, depleting neutrophils in mice reduced the antitumor effect of radiation therapy (53), which is known to activate the cGAS-STING pathway (54). As STING-S365A cells induce the neutrophil chemotactants CXCL1 and CXCL2 upon stimulation, neutrophils may contribute to the type I IFN-independent antitumor effect. Further studies are needed to determine how STING-induced NF-κB signaling drives this IFN-independent immunostimulatory effect and the subsequent antiviral and antitumor response.

Fig. 5. IFN-independent functions of STING have immunostimulatory effects. (A) BMDCs were stimulated with 75 μM DMXAA for 22 h, and CD11c+ cells were analyzed by flow cytometry with antibodies against the indicated proteins. MFI, mean fluorescence intensity. (B) Splenocytes were stimulated with 37.5 μM DMXAA for 18 h, and CD4+ T cells (CD3+ CD4+), CD8+ T cells (CD3+ CD8+), B cells (CD3− B220+), and NK cells (CD3− NK1.1+) were analyzed by flow cytometry using CD69 as the activation marker. (C) Mice were intramuscularly injected with 50 μg of OVA with or without 10 μg of cGAMP on days 0 and 7; sera and splenocytes were collected on day 14. (C) Serum ELISA for anti-OVA IgG. (D) Flow cytometric analysis of OVA-specific CD8+ T cells stained with tetramers. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Results are representative of at least two independent experiments.

The CTT of STING was dispensable for autophagy induction in stable cell lines (6) but the LC3 lipidation was weaker in BMDCs derived from the STING-ΔCTT mice (Fig. 1A), potentially due to the lower levels of native expression of STING-ΔCTT. We observed a stronger induction of NF-κB-induced genes in STING-S365A cells compared with WT cells (Fig. 4), suggesting that STING-induced IRF3 activation may inhibit NF-κB-induced gene expression. IRF3 and NF-κB synergistically induce type I IFNs, but the regulatory cross-talk between STING-induced IRF3 and NF-κB has not been well-studied. A previous study found that IFNα pretreatment in BMDMs inhibits CXCL1 and CXCL2 induction by a TLR2 ligand (55). In addition, IRF3−/− and IRF7−/− mice showed higher CXCL1 and CXCL2 expression in influenza A-infected lungs (56). We observed increased expression of several NF-κB−induced genes in STING-S365A cells, further supporting such regulatory cross-talk between the NF-κB pathway and the IFN pathway.
In summary, our study has demonstrated the type I IFN-independent antiviral and antitumor immune defense that is mediated by TBK1 recruitment to STING. Activation of the immune system by STING is critical for fighting cancer and infectious diseases, but can also lead to autoimmunity (57). Interestingly, murine models of autoimmune disease with overactive STING signaling developed inflammation even in the absence of IFNγ or IFN-κ. The authors suggest that the best role of STING in immune-related diseases will provide new insights into the physiological and pathological functions of STING, which are important for developing safe and effective treatments for human diseases.

**Materials and Methods**

**Mice.** All mice used in this study were on the C57BL/6 background. Male mice were used for HSV-1 experiments, and female mice were used for tumor experiments. STING−/−, −/−CTT mice were generated using the CRISPR/Cas9 genome engineering. CRISPR RNA and template DNA oligos were purchased from Integrated DNA Technologies (SI Appendix, Table S1). In vitro fertilization was done by the University of Texas (UT) Southwestern Transgenic Technology Center. WT and STing−/− mice were purchased from The Jackson Laboratory. In−β and 5/5 mice were provided by Rayk Behrendt, University of Technology, Dresden, Germany (60) and crossed with the Csg−/− mice generated in our laboratory (19). In−/− mice were provided by Dr. Feng, UT Southwestern. Mice were bred and maintained under specific pathogen-free conditions in the animal facility of the University of Texas Southwestern Medical Center at Dallas according to experimental protocols approved by the Institutional Animal Care and Use Committee.

**Primary Cells.** Splenocytes or bone marrow cells were filtered through a 70-μm strainer and treated with Red Blood Cell Lysis Buffer (Sigma). For generation of BMDMs, bone marrow cells were cultured in 15 ng/mL macrophage colony-stimulating factor (PeproTech) and treated with Red Blood Cell Lysis Buffer (Sigma). For generation of HSV-1 and HSV-1-GFP were propagated and titered by otherwise indicated. Primary Cells.

**HSV-1 Experiments.** HSV-1 and HSV-1-GFP were propagated and titrated by plaque assays on Vero cells (19). Male mice were infected with HSV-1 (5 x 10^6 plaque-forming units per mouse) retroorbitally under isoflurane anesthesia. For tumor infiltration measurements, Vero cells were incubated with mouse brain homogenates at serial dilutions for 1 h and then overlaid with 1.5% methylcellulose in DMEM containing 1% FBS. Seventy-two hours later, cells were fixed in methanol and formaldehyde and stained with 0.1% crystal violet. Plaques were counted to calculate viral titer from the inoculum and dilutions. For in vitro infections, cells were infected with the indicated multiplicity of infection of HSV-1 or HSV-1-GFP. DNA from HSV-1–infected cells was isolated using the DNeasy Blood & Tissue Kit (Qiagen), and the VGE was measured by qPCR and normalized by adipin (61). HSV-1–GFP–infected cells were analyzed by flow cytometry.

**LL2 Tumor Experiments.** LL2 tumor cells were grown in complete DMEM. LL2 cells (1 x 10^6) were subcutaneously injected into the flank of mice. Mice were intratumorally treated with 10 μg of GAAMP unless otherwise indicated. In VivoMab was purchased from Bio X Cell, anti-mouse PD-L1, anti-CD86, and anti-CD45 were from EMD Millipore (Millipore). Cells were labeled in 10 ng/mL Lysotracker Red (Molecular Probes) and stained with 15 ng/mL macrophage (Invitrogen). Cover-slips were coated with 50 μg/mL poly-D-lysine (Sigma) to adhere BMDCs. Stained slides were mounted on DAPI-mounting media (Vectorashield) and analyzed with an LSM700 confocal microscope. Antibodies against p-STING (S365), p-IRF3 (S396), p-β-catenin (S32), p-IκB-α (S32), p-TBK1 (S172), p-STAT1 (Y701), p-NF-kB p65 (S536), p-IKK (S172), LC3A/B, and GAPDH were from Cell Signaling; anti-mouse STING was from Proteintech; and anti–α-tubulin was from Sigma.

**Flow Cytometry and ELISA.** Cells were stained with fluorophore-labeled antibodies and fixed with 4% paraformaldehyde before analysis with a BD FACSCalibur or LSRII. The following antibodies from Biolegend were used for flow cytometry: CD45-A700, CD69-FITC/PE, CD300-FITC, NK1.1-PE, CD11b-FITC, CD11c-PB, B220-PerCP-Cy5.5, I-A/II-PE-Cy5.5, Ly6G-FITC, Ly6C-APC, CD3-APC-FITC, CD40-APC, PD-L1-APC, IgG2b-APC, CD45-A700, CD4-PAC-PE, CD86-Ig, and CD8-PerCP-Cy5.5-BV711. APC-labeled H-2K(b) SIINFEKL tetramer was generated by the NIH. IFNγ levels in cell-culture media and mouse sera were measured with an IFNγ enzyme-linked immunosorbent assay (ELISA) kit (Invivogen) according to the manufacturer’s instructions. For anti-OVA IgG ELISA, 96-well ELISA plates (Greiner Bio-One) were coated with 10 μg/mL of OVA (Sigma) and incubated with diluted sera. These plates were incubated with HRP-conjugated anti-mouse IgG (Cell Signaling), and the optical density (OD) at 450 nm was measured after developing with 3,3',5,5'-tetrabromobenzidine substrate (Thermo Scientific).

**RNA Sequencing.** RNA was isolated using an RNeasy Mini Kit (Qiagen) and sequenced by Novogene. All analyses were conducted in R (version 4.0.2). For DEG (differentially expressed gene) analysis, we used the R package edgeR and followed the user guide (62, 63). Genes with adjusted P value < 0.05 (adjusted using Benjamin-Hochberg methods) were considered as significant DEGs. The FPKM (fragments per kilobase of transcript per million mapped reads) value of each gene was generated using the R package countFPKM. The gene expression heatmap was plotted using the R package Pheatmap with the FPKM of each gene as an input. The KEGG pathway enrichment analysis was done using the R package clusterProfiler (64). Significantly enriched pathways were selected based on an adjusted P value < 0.05. Bar plots were generated using the R package ggplot2.

**qRT-PCR.** RNA was isolated from cells or tissue by using TRIzol Reagent (Invitrogen). The complementary DNA reverse-transcription kit and SYBR Green Master Mix from Applied Biosystems were used for qRT-PCR analysis according to the manufacturer’s instructions. Primers used for qRT-PCR are listed in SI Appendix, Table S2.

**Statistics.** Statistical analysis of mouse survival was performed using the Mantel–Cox test. Mouse brain viral titer was analyzed using the Kruskal–Wallis test. Other statistical analyses were performed by one-way ANOVA.

**Data Availability.** All study data are included in the article and/or SI Appendix.

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