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ARTICLE

OTUD5 promotes innate antiviral and antitumor immunity through deubiquitinating and stabilizing STING

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Stimulator of interferon genes (STING) is an adaptor protein that is critical for effective innate antiviral and antitumor immunity. The activity of STING is heavily regulated by protein ubiquitination, which is fine-tuned by both E3 ubiquitin ligases and deubiquitinases. Here, we report that the deubiquitinase OTUD5 interacts with STING, cleaves its K48-linked polyubiquitin chains, and promotes its stability. Consistently, knockout of OTUD5 resulted in faster turnover of STING and subsequently impaired type I IFN signaling following cytosolic DNA stimulation. More importantly, Lyz2-Cre *Otud5*$^{fl/Y}$ mice and CD11-Cre *Otud5*$^{fl/Y}$ mice showed more susceptibility to herpes simplex virus type 1 (HSV-1) infection and faster development of melanomas than their corresponding control littermates, indicating that OTUD5 is indispensable for STING-mediated antiviral and antitumor immunity. Our data suggest that OTUD5 is a novel checkpoint in the cGAS-STING cytosolic DNA sensing pathway.

Keywords: Antiviral innate immunity; antitumor immunity; STING deubiquitination; OTUD5

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INTRODUCTION

Innate immunity is the first line of defense against pathogenic microorganisms. The recognition of nucleic acids derived from pathogens by germline-encoded pattern recognition receptors (PRRs) initiates complex signal transduction pathways and ultimately leads to the production of type I interferon (IFN) and proinflammatory cytokines.[1] Sensing of DNA released from pathogens by circular GMP-AMP synthase (cGAS) stimulates cGAS to undergo a conformational change and induces its enzymatic activity, which is the production of cGAMP.[2,3] The endogenous second messenger cGAMP binds to the adaptor protein STING in the endoplasmic reticulum (ER) membrane.[4,5] cGAMP binding triggers STING to form oligomers through side-by-side packing. Oligomerized STING then translocates from the ER to the ER–Golgi intermediate compartment (ERGIC) and the Golgi apparatus. Upon trafficking to the Golgi, STING binds with the kinase TBK1, resulting in TBK1 activation.[6] Activated TBK1 causes the phosphorylation of the C-terminal tail region of STING, and the negatively charged surface serves as a docking site for IRF3, which is then phosphorylated and activated by TBK1.[7] Then, activated IRF3 dimerizes and translocates into the nucleus to modulate the expression of IFN-β.[8]

The cGAS-STING pathway not only mediates protective immune defense against infection by a large variety of DNA-containing pathogens but also detects tumor-derived DNA and supports intrinsic antitumor immunity through promoting the production of type I interferon.[9] Type I interferon is the bridge between innate immunity and adaptive immunity. Type I interferon produced by antigen-presenting dendritic cells in the tumor microenvironment can activate innate immunity and promote T cell activation and cross-infiltration.[10] However, aberrant activation of the cGAS-STING pathway by self-DNA can also lead to autoimmune and inflammatory disease.[11] Therefore, effective and tightly regulated cGAS-STING signaling is critical for facilitating antiviral and antitumor immunity and for preventing autoimmunity. However, the regulatory mechanisms of cGAS-STING are not yet completely known.

Numerous studies have shown that ubiquitination, one of the most common posttranslational modifications (PTMs), is indispensable for fine-tuning the cGAS-STING signaling pathway.[12] Ubiquitination is a reversible process controlled by both E3 ubiquitin ligases and deubiquitinases (DUBs). DUBs mainly cleave ubiquitin molecules from ubiquitin-conjugated proteins or precursor proteins by hydrolyzing the carboxyl-terminus ester, peptide, or isopeptide bonds. Currently, many of the ~100 DUBs have been identified and divided into six families: the ubiquitin C-terminal hydrolase family (UCHs), Machado–Joseph disease protein proteases (MJDs), JAMM/MPN domain-associated metallopeptidases (JAMMs), monocyte chemotactic protein-induced proteins (MCPIPs), ovarian tumor (OTU) proteases, and ubiquitin-specific proteases (USPs).[13] Previous studies have demonstrated that many E3 ubiquitin ligases regulate the activity of STING. RNF5 promotes K48-linked polyubiquitination and the subsequent degradation of STING.[14] AMFR and TRIM56 facilitate K27-linked

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and K63-linked polyubiquitination of STING, leading to enhanced interactions between STING and TBK1, respectively.[15] Correspondingly, several DUBs have been found to modulate the deubiquitination of STING. USP18 was reported to recruit USP20 for the cleavage of K48-linked polyubiquitin chains from STING and prevent its proteasomal-dependent degradation.[16] USP13 and USP49 were found to remove K27- and K63-linked polyubiquitin chains from STING, which reduced the interaction between STING and TBK1, respectively.[17,18] These studies suggest that DUBs also have an important role in modulating the activity of STING. However, the identified DUBs that regulate STING all belong to the USP family. Whether DUBs in other families are also involved in the regulation of STING-mediated innate immunity is currently unclear.

As the second largest human family of DUBs, 18 members of the ovarian tumor (OTU) DUB family are found in the human genome, 16 of which have been annotated as active DUBs that regulate important cell signaling cascades.[19] OTUD7B, OTULIN, and A20 have been demonstrated to participate in NF-κB signaling.[20–22] OTUD5 regulates interferon signaling by downregulating TRAF3 K63-linked polyubiquitination,[23] OTUD2/YOD1 and VCPIP regulate p97-mediated processes, and OTUB1 is involved in the DNA damage response.[24,25] However, whether the OTU family DUBs are involved in the regulation of cGAS-STING signaling has not been investigated.

In this study, we found that OTUD5 cleaves the K48-linked polyubiquitin chains from STING and that this cleavage is dependent on the enzymatic activity of OTUD5. Consequently, OTUD5 promotes the stability of STING and type I interferon signaling during viral infection. Consistent with the in vitro results, mice with a conditional knockout of OTUD5 in myeloid cells or dendritic cells displayed less resistance to herpes simplex virus type 1 (HSV-1) infection and faster progression of melanoma than their respective control littermates. Our study indicates that OTUD5 has an indispensable role in STING-mediated antiviral and antitumor immunity.

RESULTS

OTUD5 regulates the deubiquitination of STING

Previous studies have demonstrated that STING is extensively modified by various types of ubiquitin chains, and these modifications are essential for its stability and activation.[14,15,26] Ubiquitination is a reversible reaction catalyzed by both E3 ubiquitin ligases and DUBs. As mentioned above, DUBs are divided into several families, within which the OTU family has the second largest numbers. Even though there are a series of DUBs from the USP family with known involvement in modulating STING deubiquitination,[16] whether other DUBs from the OTU family also affect the deubiquitination of STING and subsequently regulate STING-mediated innate immunity is unknown. To search for DUBs from the OTU family that participate in STING deubiquitination, we screened 15 OTU family DUBs and found that OTUD5 significantly decreased the ubiquitination level of STING (Supplementary Fig. 1A, B).

Given that OTUD5 is a DUB, we next examined whether its impact on STING ubiquitination depends on its enzymatic activity. We constructed a C224S mutant (mutation of cysteine to serine at position 224), which lacks deubiquitination activity.[23,27] We cotransfected Myc-STING with Flag-OTUD5 or its enzyme inactive mutant C224S into HEK293T cells; we observed that WT OTUD5 but not mutant C224S led to a significant reduction in STING ubiquitination (Fig. 1a), which indicated that OTUD5 enzymatic activity was indispensable for the deubiquitination of STING. To further validate this notion, we performed an in vitro deubiquitination assay with recombinant Flag-OTUD5 protein or C224S. Consistent with the results from the deubiquitination assay in HEK293T cells, recombinant WT OTUD5 protein, but not the mutant C224S protein, was able to decrease STING ubiquitination (Fig. 1b). Furthermore, we investigated whether OTUD5 could mediate STING deubiquitination under physiological conditions. We observed that siRNA knockdown of OTUD5 expression in primary peritoneal macrophages led to increased endogenous STING ubiquitination after HSV-1 infection compared to what was observed in control siRNA-transfected macrophages (Supplementary Fig. 1C). Consistent with the siRNA knockdown results, following HSV-1 infection, peritoneal macrophages from Lyz2-Cre *Otud5*$^{fl/Y}$ mice also showed an enhanced level of STING ubiquitination compared to macrophages from *Otud5*$^{fl/Y}$ mice (Fig. 1c). Taken together, we demonstrated that OTUD5, through its enzymatic activity, regulates the ubiquitination level of STING.

OTUD5 interacts with STING

To investigate whether OTUD5 regulates STING deubiquitination by forming a complex with it, we first examined whether OTUD5 could interact with STING using a co-IP assay. We transfected Flag-OTUD5 or OTUD5 C224S and Myc-STING into HEK293T cells and observed an association between OTUD5 and STING through reciprocal coimmunoprecipitation assays (Fig. 1d, e). Notably, C224S could interact with STING in a fashion similar to that of WT OTUD5, indicating that the enzymatic activity of OTUD5 is not required for the interaction with STING (Fig. 1d, e). Next, we performed an in vitro binding assay with purified Flag-OTUD5 or C224S and V5-STING, and the results showed that both WT OTUD5 and C224S bound directly to STING (Fig. 1f). Furthermore, we found that endogenous STING and OTUD5 could form a complex upon HSV-60 transfection (Fig. 1g). Immunofluorescence and confocal microscopy analysis showed that a substantial portion of OTUD5 colocalized with STING (Fig. 1h, i), further confirming their interaction. Domain mapping analysis revealed that the cyclic dinucleotide-binding domain (amino-acid residues 153–339) of STING and the intact region of OTUD5 from amino acids 1–220 were necessary for STING and OTUD5 interaction (Fig. 1j, k). Overall, these observations indicated that OTUD5 regulates the ubiquitination of STING by directly interacting with STING.

OTUD5 promotes STING stability

As different forms of ubiquitin chains have distinct roles in the regulation of protein function, we next investigated the type of STING ubiquitination modulated by OTUD5. We cotransfected HEK293T cells with Myc-STING, HA-Ub or its site mutants with or without Flag-OTUD5. The results showed that OTUD5 could significantly downregulate the ubiquitination level of STING in cells transfected with WT ubiquitin and the ubiquitin mutant K48 (Fig. 2a). Importantly, OTUD5 could not decrease the ubiquitination level of STING in cells transfected with the ubiquitin mutant K48R, but the levels were decreased in K63R-transfected cells, indicating that OTUD5 cleaves the K48-linked ubiquitination of STING rather than other types of polyubiquitin chains (Fig. 2a). Furthermore, knockout of OTUD5 in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages followed by the introduction of HSV-1 infection also led to increased K48-linked polyubiquitination rather than K63-linked polyubiquitination of endogenous STING compared to the levels observed in *Otud5*$^{fl/Y}$ macrophages (Fig. 2b). As STING mainly interacts with OTUD5 through the CBD region, we speculate that OTUD5 may regulate the ubiquitination of lysine residue(s) in the CBD or near the CBD of STING. We performed deubiquitination assays with WT STING and its various KR mutants, in which the lysine residues in the CBD or near the CBD have been individually mutated to arginine. The results showed that the STING mutant K347R was resistant to OTUD5-mediated deubiquitination, suggesting that OTUD5 mainly deubiquitinates STING at K347 (Fig. 2c). As K48-linked ubiquitination signals the substrate for proteasomal degradation, we next examined whether OTUD5 could regulate the stability of STING. Overexpression of WT Flag-OTUD5 in HEK293T cells increased the Myc-STING protein level in a

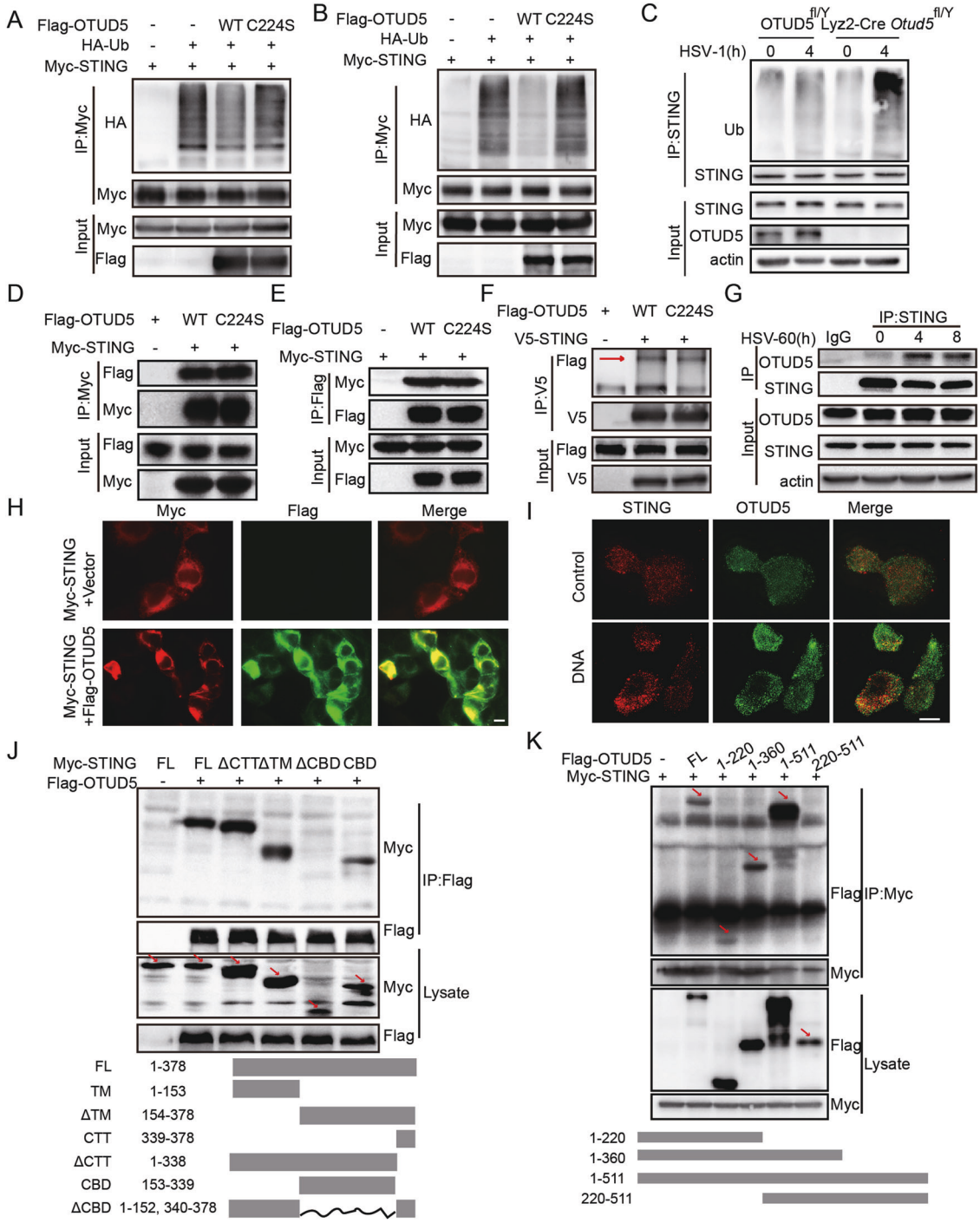

Fig. 1 OTUD5 deubiquitinates STING. **a** Myc-STING and HA-ubiquitin (HA-Ub) were transfected into HEK293T cells together with Flag-OTUD5 or C224S. Cells were pretreated with MG132 for 2 h, and cell lysates were subjected to Co-IP with anti-Myc Ab, which was followed by western blotting with an anti-HA Ab. **b** HEK293T cells were transiently transfected with Myc-STING and HA-Ub, and ubiquitinated STING was enriched with an anti-Myc Ab. In vitro synthesized OTUD5 or C224S was incubated together with ubiquitinated STING in deubiquitylation assay buffer at 37 °C for 1 h. STING ubiquitination was analyzed by western blotting with an anti-HA Ab. **c** *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages were infected with HSV-1 (MOI = 10) and harvested at the indicated times. Ubiquitinated STING was enriched with an anti-STING Ab and then was detected with an anti-ubiquitin (Ub) Ab. **d**, **e** Co-IP was performed with lysates from HEK293T cells expressing Myc-STING and Flag-OTUD5 or C224S. **f** V5-STING and Flag-OTUD5 or C224S were obtained by in vitro transcription and translation. The interaction between STING and OTUD5 or C224S was assayed by Co-IP with an anti-V5 Ab and western blotting with an anti-Flag Ab. Targeted protein bands are indicated by red arrows. **g** Co-IP was performed in lysates of peritoneal macrophages transfected with HSV-60 for the indicated times. **h** Colocalization of Myc-STING (red) with Flag-OTUD5 (green) in HEK293 cells. Scale bar, 20 μM. **i** Colocalization of endogenous STING (red) with OTUD5 (green) in cultured macrophages stimulated with 10 μM cytosolic DNA. **j** Myc-STING or its truncation mutants were transfected into HEK293T cells together with Flag-OTUD5, and cell lysates were subjected to Co-IP with an anti-Flag Ab, which was followed by western blotting with an anti-Myc Ab. Targeted protein bands are indicated by red arrows. **k** Flag-OTUD5 or its truncation mutants were transfected into HEK293T cells together with Myc-STING, and cell lysates were subjected to Co-IP with an anti-Myc Ab, which was followed by western blotting with an anti-Flag Ab. Targeted protein bands are indicated by red arrows. Similar results were obtained in three independent experiments

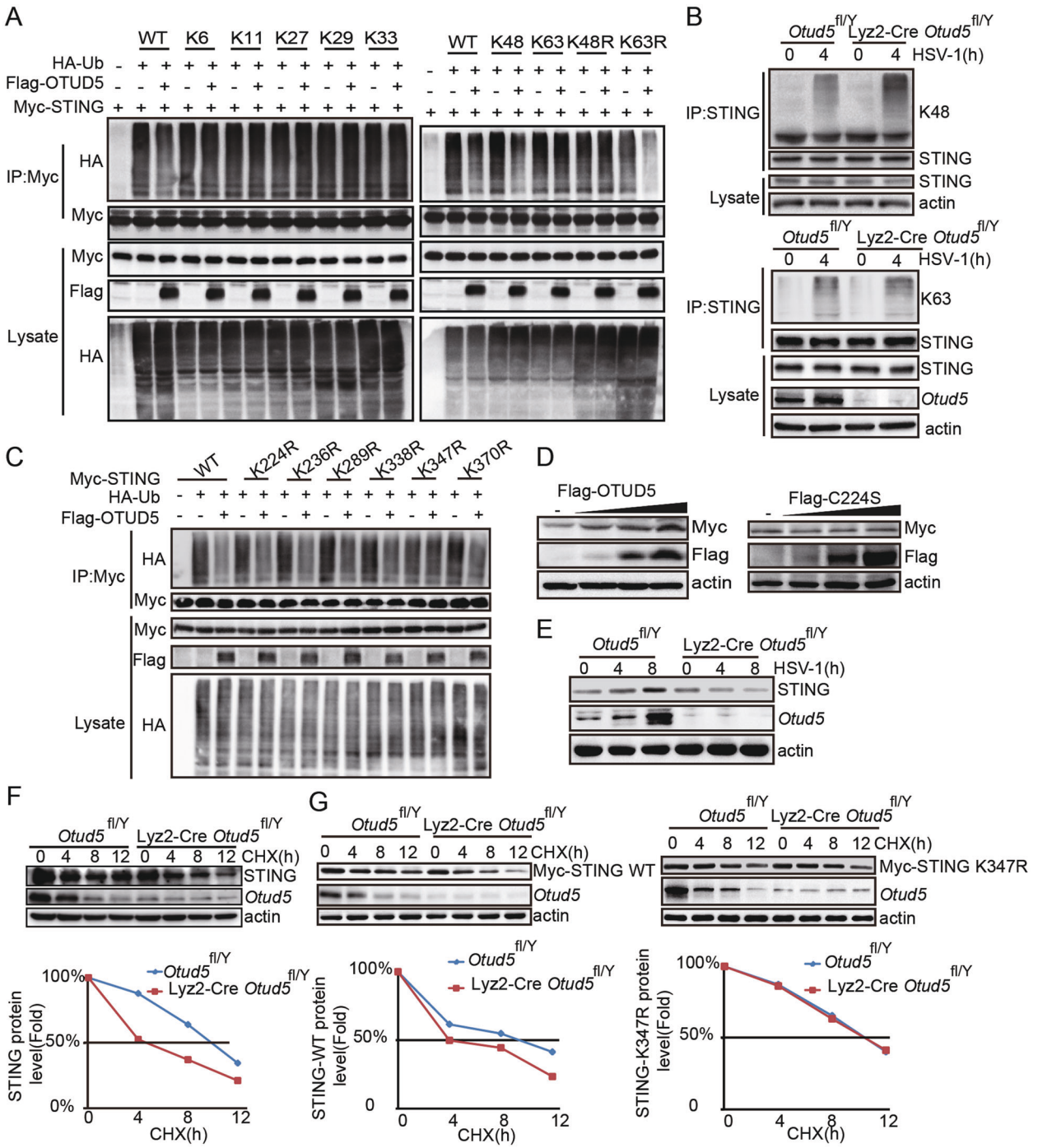

Fig. 2 OTUD5 regulates STING stability. **a** Myc-STING, HA-ubiquitin (HA-Ub), or its lysine residue mutants were transfected into HEK293T cells with or without Flag-OTUD5. Cells were pretreated with MG132 for 2 h, and Co-IP assays were performed. **b** *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages were pretreated with MG132 for 2 h, which was followed by infection with HSV-1 for 4 h; then an IP was performed with an anti-STING Ab, and western blotting was performed with a K48 or K63 antibody. **c** HEK293T cells (1×10^6) were transfected with Myc-STING or its mutants, HA-ubiquitin, with or without Flag-OTUD5. Cells were pretreated with MG132 for 2 h, and Co-IP assays were performed. **d** Myc-STING was transfected into HEK293T cells together with increasing amounts of Flag-OTUD5 or C224S. STING protein levels were detected by western blotting. **e** *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages were infected with HSV-1 for the indicated times, and STING protein levels were detected by western blotting. **f** *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages were infected with HSV-1 for 4 h and then were treated with cycloheximide (CHX) for the indicated times. STING protein levels were detected by western blotting. Quantification of STING protein levels is shown relative to β-actin levels. **g** *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages were transfected with WT Myc-STING or the Myc-STING mutant K347R, which was followed by HSV-1 infection for 4 h; then, cells were treated with cycloheximide (CHX) for the indicated times. Myc-STING protein levels were detected by western blotting. Quantification of Myc-STING protein levels is shown relative to β-actin levels. Similar results were obtained in three independent experiments

dose-dependent manner, while the Myc-STING protein level was not affected in C224S-transfected cells (Fig. 2d). Consistent with the overexpression results, knockout of OTUD5 in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages followed by HSV-1 infection resulted in a substantial decrease in the protein level of STING compared with the level in *Otud5*$^{fl/Y}$ macrophages (Fig. 2e). Similarly, knockout of OTUD5 in RAW264.7 macrophages with CRISPR-Cas9 technology also decreased STING protein levels after HSV-1 infection (Supplementary Fig. 2A). To prove that OTUD5 affects STING stability by itself, we treated the cells with the de novo protein synthesis inhibitor cycloheximide (CHX) and measured the degradation of STING. We found that the half-life of STING in macrophages was significantly shortened in macrophages with OTUD5 knocked out (Fig. 2f), as shown in OTUD5 knockout RAW264.7 macrophages (Supplementary Fig. 2B). As a control, we showed that the half-life of RIG-I was not affected in OTUD5-deficient macrophages (Supplementary Fig. 2C). To further prove that OTUD5 affects STING degradation by regulating its deubiquitination, we transfected WT Myc-STING or its mutant K347R into *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages to measure the degradation of WT STING and the K347R mutant. We found that the half-life of WT STING was shortened in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages, whereas the deletion of OTUD5 did not affect the turnover rate of the STING mutant K347R (Fig. 2g); these data indicate that the stability of the STING mutant K347R is not regulated by OTUD5. Taken together, these results demonstrated that OTUD5 promotes the stability of STING by cleaving the K48-linked polyubiquitin chains from STING K347.

OTUD5 positively regulates the STING-mediated type I interferon response

As STING serves as the critical adaptor protein for type I interferon signaling to protect cells against cytosolic DNA, we next examined whether OTUD5 regulates the STING-mediated type I interferon response. We first transfected primary macrophages with mouse *Otud5* siRNA and observed that the siRNA-mediated knockdown of *Otud5* expression significantly reduced *Ifnb1* mRNA expression and IFN-β production upon stimulation with ISD and cGAMP or infection with HSV-1 (Supplementary Fig. 3A, B). gRNA-mediated knockout of OTUD5 in RAW264.7 macrophages also decreased *Ifnb1* mRNA expression upon stimulation with ISD and cGAMP or infection with HSV-1 (Supplementary Fig. 3C). Furthermore, we transfected THP-1 cells with human OTUD5 siRNA and obtained the same results upon stimulation with ISD and cGAMP or infection with HSV-1 (Supplementary Fig. 3D), indicating that the function of OTUD5 in the regulation of the type I interferon response is not species-specific.

To definitively confirm the function of OTUD5 in the STING-mediated type I interferon response, we prepared primary macrophages from Lyz2-Cre *Otud5*$^{fl/Y}$ mice and their littermates. Consistent with the siRNA knockdown results, knockout of OTUD5 in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages reduced the *Ifnb1* mRNA level after infection with HSV-1 or stimulation with HSV-60, ISD, and cGAMP compared with that of *Otud5*$^{fl/Y}$ macrophages (Fig. 3a). Congruently, the expression of *Ccl5* and *Cxcl10*, which are genes that are downstream of IFN-β signaling, was also decreased in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages after infection with HSV-1 or stimulation with HSV-60 and ISD (Fig. 3b). We further found that secretion of IFN-β in response to HSV-1 infection, stimulation with cGAMP or ISD, and HSV-60 transfection was significantly impaired in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages (Fig. 3c). OTUD5 is also called DUBA, which has been shown to negatively regulate IFN-β expression downstream of RLR and TLR4 signaling through cleavage of K63-linked polyubiquitin chains on TRAF3.[23,27] Consistent with this report, we found that the *Ifnb1* mRNA level was increased in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages induced by SeV and VSV infection and LPS stimulation (Fig. 3d). Taken together, these data demonstrated that OTUD5 positively regulates cytosolic DNA-induced IFN-β production.

OTUD5 positively regulates STING-mediated signaling

To assess changes in STING-mediated signaling cascades in response to cytosolic DNA stimulation, we analyzed changes in the phosphorylation of TBK1 and IRF3, which are downstream of STING activation. We observed that the siRNA-mediated knockdown of *Otud5* expression in mouse peritoneal macrophages significantly reduced the phosphorylation of TBK1 and IRF3 upon infection with HSV-1 or transfection with ISD (Supplementary Fig. 4A). Knockout of *Otud5* in RAW264.7 macrophages also led to decreased phosphorylation of TBK1 and IRF3 upon infection with HSV-1 or transfection with ISD (Supplementary Fig. 4B). Similarly, siRNA-mediated OTUD5 knockdown in THP-1 cells also attenuated the phosphorylation of TBK1 and IRF3 upon infection with HSV-1 and transfection with ISD (Supplementary Fig. 4C).

To definitively confirm the function of OTUD5 in STING-mediated phosphorylation of TBK1 and IRF3, we prepared primary macrophages from Lyz2-Cre *Otud5*$^{fl/Y}$ mice and *Otud5*$^{fl/Y}$ mice. The knockout of OTUD5 in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages led to a decrease in TBK1 and IRF3 phosphorylation after infection with HSV-1 (Fig. 4a). Similarly, ISD-, HSV-60-, and cGAMP-induced phosphorylation of IRF3 and TBK1 was also decreased in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages compared to *Otud5*$^{fl/Y}$ macrophages (Fig. 4a). Consistent with the negative role of OTUD5 in RLR and TLR4 signaling, SeV- and LPS-induced phosphorylation of IRF3 and TBK1 was increased in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages (Fig. 4b). As IRF3 phosphorylation led to the dimerization of IRF3, we then examined the changes in IRF3 dimerization. Consistent with the above finding, ISD- and cGAMP-induced IRF3 dimerization was greatly decreased in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages (Fig. 4c). To investigate how the function of K347 ubiquitination was regulated by OTUD5, we transfected Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages with WT STING and its mutant K347R and measured TBK1 and IRF3 phosphorylation after HSV-1 infection. We found that overexpression of WT STING in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages substantially increased HSV-1-induced phosphorylation of IRF3 and TBK1 as well as *Ifnb1* mRNA levels (Fig. 4d, e). Notably, overexpression of the STING mutant K347R in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages further increased HSV-1-induced phosphorylation of IRF3 and TBK1 and *Ifnb1* mRNA levels compared with WT STING (Fig. 4d, e). Taken together, these data indicated that OTUD5 regulates cytosolic DNA-induced TBK1 and IRF3 phosphorylation to regulate IFN-β production.

OTUD5 is required for host defense against HSV-1 infection

As OTUD5 has an important role in the HSV-1-induced type I interferon response, we next investigated whether OTUD5 regulates HSV-1 infection. We first prepared primary macrophages from Lyz2-Cre *Otud5*$^{fl/Y}$ mice and their littermates following infection with HSV-1. Plaque assays showed that Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages exhibited significantly increased rates of HSV-1 replication compared with those of *Otud5*$^{fl/Y}$ macrophages (Fig. 5a). As a control, we also infected macrophages with VSV, an RNA virus sensed by RLR. Consistent with the function of OTUD5 in negatively regulating RLR signaling,[27] VSV infection-induced phosphorylation of TBK1 and IRF3 was increased in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages (Fig. 5b). VSV titers and VSV-G protein levels were decreased in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages (Fig. 5b), further confirming the function of OTUD5 in negatively regulating RLR signaling.

To examine the role of OTUD5 in regulating HSV-1 infection in vivo, we infected *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ mice with HSV-1 peritoneally and monitored their survival rates. We observed that Lyz2-Cre *Otud5*$^{fl/Y}$ mice were more susceptible to HSV-1 infection than *Otud5*$^{fl/Y}$ mice (Fig. 5c). In addition, the copy number of HSV-1 genomic DNA and viral titers in the brain were significantly higher in Lyz2-Cre *Otud5*$^{fl/Y}$ mice than they were in *Otud5*$^{fl/Y}$ mice (Fig. 5d), which was further confirmed by the immunohistochemistry (IHC) results of brain sections stained with

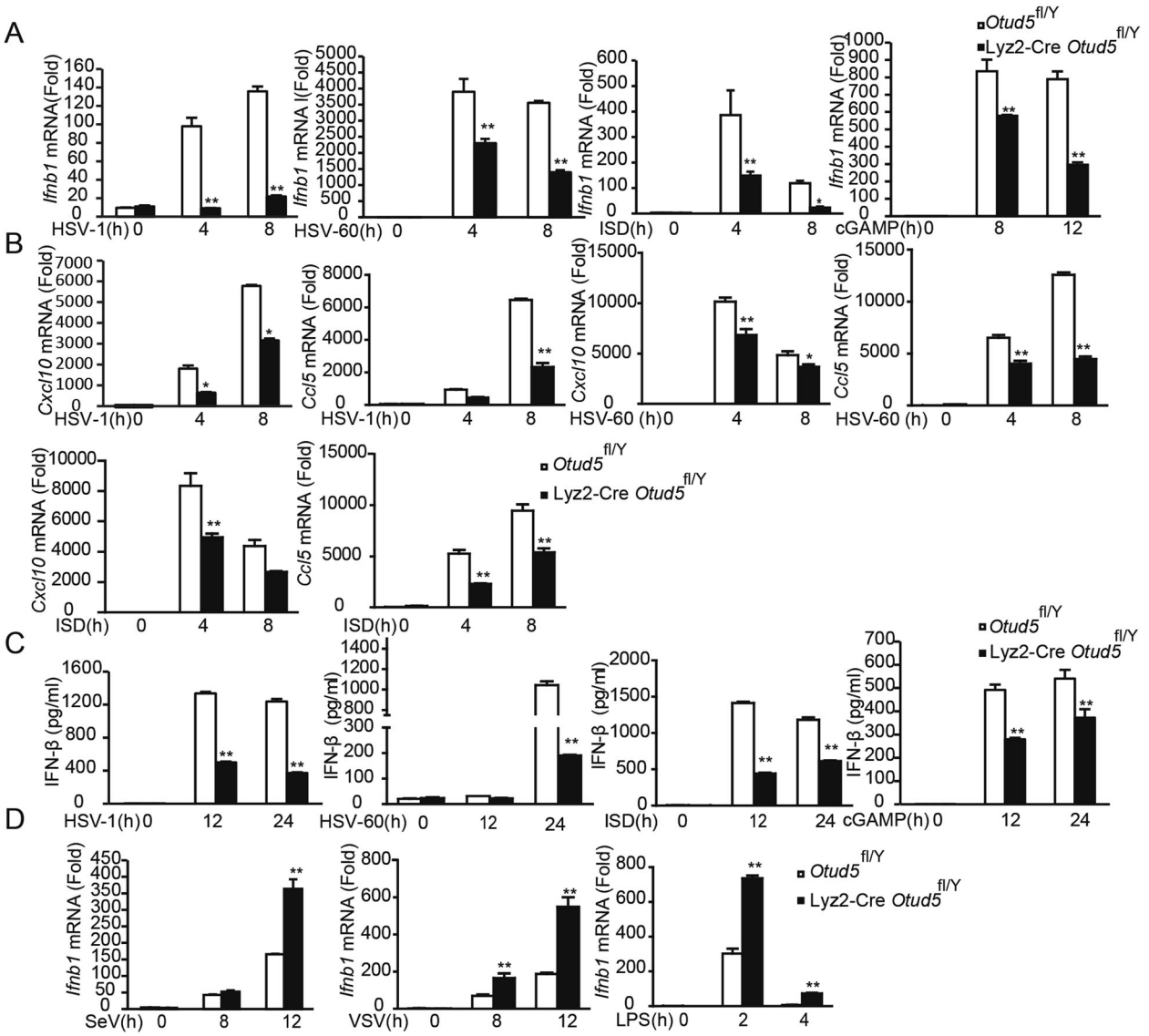

Fig. 3 OTUD5 positively regulates the STING-mediated type I interferon response. **a** qPCR of *Ifnb1* mRNA in *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages infected with HSV-1 stimulated with cGAMP, ISD, or HSV-60 for the indicated time points. **b** qPCR of *Ccl5* and *Cxcl10* mRNA in *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages infected with HSV-1 or stimulated with ISD or HSV-60 for the indicated time points. **c** ELISA of IFN-β production in culture supernatant from *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages infected with HSV-1 stimulated with ISD, HSV-60, or cGAMP. **d** qPCR of *Ifnb1* mRNA in *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages stimulated with LPS or infected with VSV and SeV for the indicated time points. *$p < 0.05$ and **$p < 0.01$

an HSV-1 protein ICP8-specific antibody (Fig. 5e). Taken together, our data indicated that OTUD5 is indispensable for the host antiviral response.

OTUD5 regulates radiation-mediated antitumor immunity

To explore the possible antitumor effects of the OTUD5/STING axis, we first performed IHC staining for STING and OTUD5 in an independent cohort of non-small cell lung cancer tissues (NSCLC) ($n = 73$) and corresponding noncancerous lung tissues ($n = 17$). STING and OTUD5 were found to be expressed in the cytoplasm (Fig. 6a). We quantified STING and OTUD5 expression in the sections of lung cancer patients and found that both STING and OTUD5 protein levels were higher in noncancerous lung tissues than they were in lung tumors (Fig. 6a, b). Importantly, a very good correlation between OTUD5 and STING protein expression was detected in both lung tumors and noncancerous lung tissues (Fig. 6c). These data suggest that OTUD5 may regulate STING stability in lung cancer and that the OTUD5/STING axis may have antitumor roles.

Previous studies have demonstrated that ionizing radiation-induced type I interferon-dependent antitumor immunity is dependent on STING.[9] Ionizing radiation led to the release of DNA fragments from cancer cells, which were recognized and resulted in cGAS-STING signaling in dendritic cells (DCs). The activation of DCs produces type I interferon and recruits cytotoxic T cells to repress cancer development. As OTUD5 regulates the stability of STING and subsequently affects type I interferon signaling in macrophages, we next explored whether OTUD5 in DCs modulates the ionizing radiation-mediated antitumor effect.

To determine whether OTUD5 regulates STING in DCs, we prepared BMDCs from CD11-Cre *Otud5*$^{fl/Y}$ and *Otud5*$^{fl/Y}$ mice and stimulated them with ISD and cGAMP. Similar to the function of OTUD5 in macrophages, we observed that knocking out OTUD5 in BMDCs attenuated IFN-β secretion upon stimulation with ISD and cGAMP (Supplementary Fig. 5A). Furthermore, ISD-, cGAMP-, HSV-60-, and HSV-1 infection-induced phosphorylation of IRF3 and TBK1 was also decreased in CD11-Cre *Otud5*$^{fl/Y}$ DCs

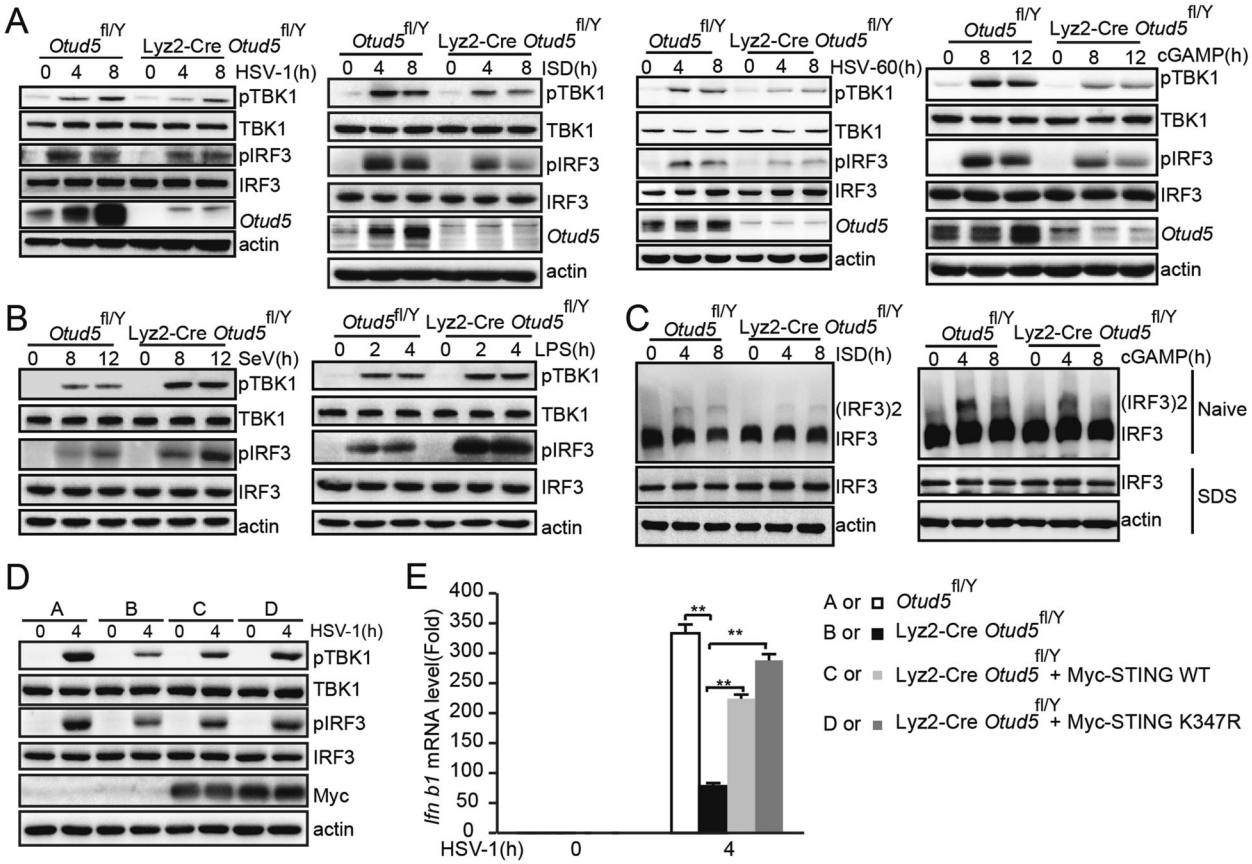

Fig. 4 OTUD5 positively regulates STING-mediated signaling. **a** Western blot of phosphorylated (p) and total IRF3 and TBK1 in *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages stimulated with ISD, HSV-60 transfection and cGAMP (50 mg/ml) or infected with HSV-1 (MOI = 10) for the indicated time points. **b** Western blot of phosphorylated (p) and total IRF3 and TBK1 in *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages stimulated with LPS or infected with SeV. **c** Native PAGE analysis of IRF3 dimerization in *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages stimulated with ISD or cGAMP for the indicated time points. **d** Western blot of phosphorylated (p) and total IRF3 and TBK1 in *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages or Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages overexpressing WT STING or the STING mutant K347R, which was followed by infection with HSV-1 (MOI = 10). **e** qPCR analysis of *Ifnb1* mRNA in *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages or Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages overexpressing WT STING or STING mutant K347R, followed by infection with HSV-1 (MOI = 10). $^{**}p < 0.01$. Similar results were obtained in three independent experiments

(Supplementary Fig. 5B). These data suggested that OTUD5 in DCs could regulate cytosolic DNA-induced type I interferon signaling.

To directly investigate the function of OTUD5 in the radiation-mediated tumor antitumor immune response, we implanted B16-Luc cells into CD11-Cre *Otud5*$^{fl/Y}$ and *Otud5*$^{fl/Y}$ mice and then treated the mice with radiation (Gy = 25); then, we examined tumor growth and IFN-β production in the tumors. We found that the production of IFN-β in tumors was significantly attenuated in the absence of OTUD5 in the mice 3 days after radiation (Fig. 6d). Furthermore, the CD11-Cre *Otud5*$^{fl/Y}$ mice showed a significant increase in B16-Luc tumor growth after radiation treatment, which was monitored and quantified using bioluminescence (Fig. 6e). These above results indicate that OTUD5 can participate in the antitumor immunity of radiation therapy by regulating STING.

DISCUSSION

cGAS-STING signaling has an important role in innate immunity. STING is a point of confluence that integrates abnormal cytoplasmic DNA detection with upstream signals, which results in TBK1 and IRF3 activation, and finally induces the production of type I IFN and proinflammatory cytokines.[28] However, dysregulation of STING signaling has been shown to be involved in cancer and autoimmune diseases.[11] Therefore, the intensity and duration of STING signaling is dynamically adjusted in a multilayered and highly ordered manner to maintain immune homeostasis.

It is well established that ubiquitination/deubiquitination serves to fine-tune the STING activity response to DNA stimulation.[17,29] In this study, we screened OTU family DUBs and found that OTUD5 could remove STING K48-linked ubiquitination and further stabilize STING. To explore the physiological functions of OTUD5, we established conditional *Otud5* knockout mice. We found that knockout of *Otud5* specifically in macrophages impaired host defense against HSV-1 infection. Furthermore, we found that knockout of *Otud5* specifically in DCs impaired antitumor immunity after radiation, which involved a decrease in the production of IFN in tumors and an increase in tumor growth in CD11-Cre *Otud5*$^{fl/Y}$ mice. These data suggested that OTUD5 has an indispensable role in STING-mediated antiviral and antitumor immunity.

Several DUBs, such as CYLD, USP44, and USP18/USP20, have been reported to remove K48-linked polyubiquitination from STING and regulate STING protein stability. Using an in vitro binding assay, we found that OTUD5 interacts with STING directly. CYLD was found to interact selectively with K48-linked poly-ubiquitin chains on STING in a ubiquitination-dependent manner to regulate STING stability.[30] USP18 was found to interact with STING after viral infection and recruits USP20 to deconjugate K48-linked polyubiquitin chains to regulate STING stability.[16] We also demonstrated that OTUD5 expression was induced by the activation of DNA recognition-mediated signaling pathways, such as HSV-1 infection. Thus, our data suggested that OTUD5 may work as a major feedback regulator to control STING

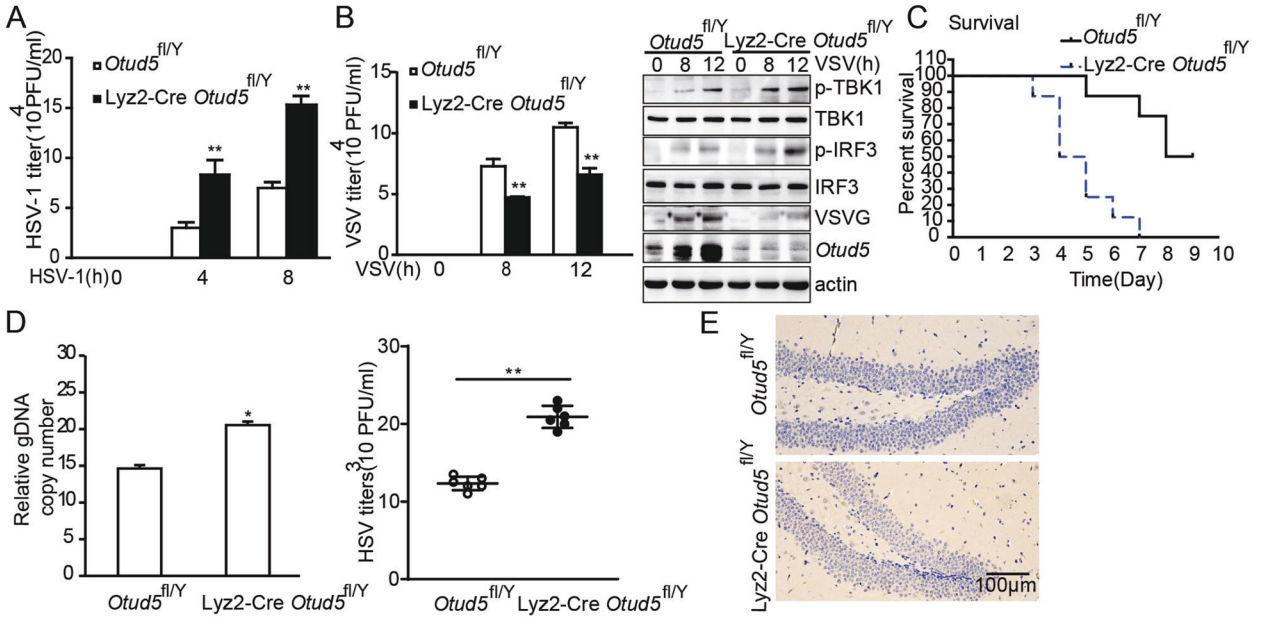

Fig. 5 OTUD5 is required for host defense against HSV-1 infection. **a**, **b** *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages were infected with HSV-1 or VSV, and HSV-1 or VSV titers were measured using plaque assays. VSV-G protein levels were detected in macrophages of *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages infected with VSV. **c** *Otud5*$^{fl/Y}$ and Lyz2-Cre *Otud5*$^{fl/Y}$ mice were infected with HSV-1 (1×10^8 pfu per mouse), and the survival rates were monitored for 9 days (8 mice per group). **d** The HSV-1 genomic DNA copy numbers and the HSV-1 titers in the brains of HSV-1-infected mice (1×10^8 pfu per mouse) were measured. **e** Brains of the HSV-1-infected mice were harvested for immunohistochemistry (IHC) analysis with an antibody against HSV-1 ICP8 protein. Tissue sections were visualized by microscopy. Similar results were obtained in three independent experiments. *$p < 0.05$ and **$p < 0.01$

deubiquitination and stability. How OTUD5 coordinates with these other DUBs to regulate the K48-linked ubiquitination and protein stability of STING needs further investigation.

OTUD5 is also called DUBA, and it has been shown to have important roles in immunity. Studies have reported that DUBA can selectively cleave the K63-linked polyubiquitin chains on TRAF3, resulting in the attenuation of type I interferon production downstream of RLR and TLR signaling.[23] Here, we confirmed the study using Lyz2-Cre *Otud5*$^{fl/Y}$ mice. We found that macrophages from Lyz2-Cre *Otud5*$^{fl/Y}$ mice showed increased IFN-β production and phosphorylation of TBK1 and IRF3 upon infection with SeV or stimulation with LPS. Importantly, VSV replication in Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages was greatly decreased compared to the levels in WT macrophages. DUBA is also reported to be a negative regulator of IL-17 production in T cells. Mechanistically, DUBA accumulation in activated T cells leads to the stabilization of URB5, which then ubiquitinates RORγt in response to TGF-β signaling.[31] In the present study, we found that OTUD5 can target STING and regulate its ubiquitination and stabilization to regulate cytosolic DNA-mediated innate immunity. Thus, we identified a novel function for OTUD5: it can target different molecules to regulate various immune responses.

In summary, we confirmed that the deubiquitinating enzyme OTUD5 can directly bind to STING to regulate its K48-linked deubiquitination, which affects STING protein stability and further regulates the role of STING in antiviral immunity and antitumor immunity. Given that cGAS/STING signaling has an important role in antiviral and antitumor immunity and autoimmune diseases, OTUD5 may have therapeutic potential for the prevention and treatment of human diseases with aberrant DNA signaling.

MATERIALS AND METHODS

Lead contact and materials availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by Dr Chengjiang Gao (cgao@sdu.edu.cn). All unique reagents generated in this study are available upon the completion of a Materials Transfer Agreement.

Experimental model and subject details

Reagents. LPS (*Escherichia coli* 055:B5) was purchased from Sigma-Aldrich. cGAMP was purchased from InvivoGen. LPS and cGAMP were used at final concentrations of 100 ng/ml and 50 μg/ml, respectively.

Antibodies were purchased as follows: rabbit anti-c-Myc was from Bethyl; rabbit anti-Flag was from ThermoFisher Scientific; rabbit anti-HA was from Rockland; rabbit anti-OTUD5 was from Abcam; rabbit anti-STING, anti-IRF3, anti-pIRF3, anti-TBK1, and anti-pTBK1 were from Cell Signaling Technology; mouse anti-FLAG (M2) was from Sigma-Aldrich; horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG were from Calbiochem.

Cells and viruses. Human HEK293T and HEK293 cells were obtained from American Type Culture Collection. The cells were cultured at 37 °C under 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Sendai virus (SeV) was purchased from the China Center for Type Culture Collection (Wuhan University, China). Vesicular stomatitis virus (VSV), VSV-GFP, and herpes simplex virus 1 (HSV-1) were kindly provided by H. Meng (Institute of Basic Medicine, Shandong Academy of Medical Sciences, China).

Plasmids and siRNA. The coding regions of human OTUD5 or human STING were amplified from human cDNA and subcloned into pCDNA3.1 or pcMV-N-Myc vectors. UCHL1, UCHL3, UCHL5, OTUB1, and YOD1 were kindly provided by Prof. Bing Li (Shanghai Jiao Tong University, China). The coding regions of BAP1, OTUB2, OTUD3, and OTUD4 were purchased from Origene. Sequences for other DUBs were purchased from GeneCopoeia. HA-Ub and other plasmids were kept in our laboratory. Three small interfering RNAs (siRNAs) against OTUD5 were designed, synthesized, and purified by RiboBio. The target sequences were as follows: hOTUD5:

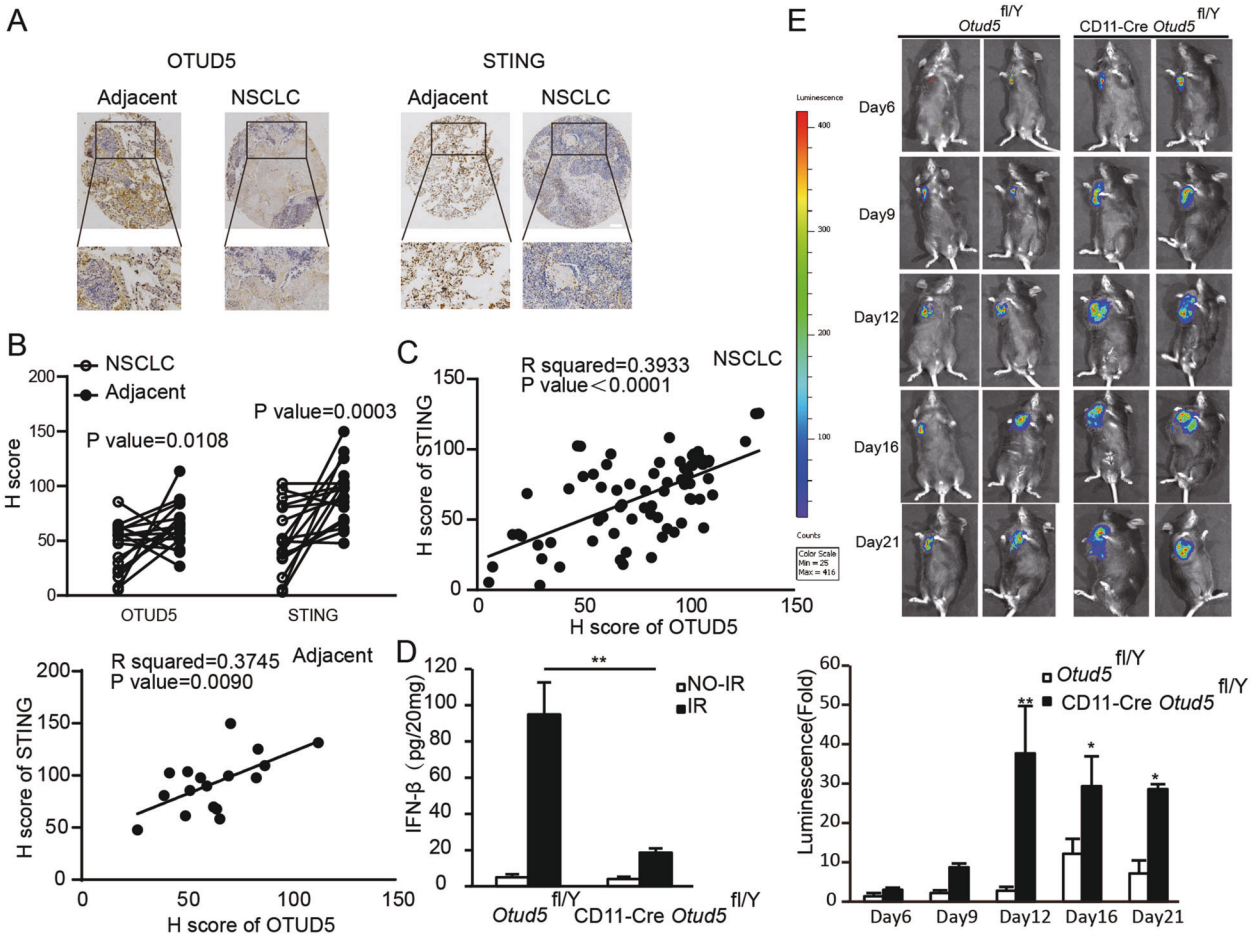

Fig. 6 OTUD5 regulates radiation-mediated antitumor immunity. **a** Representative images of IHC staining for STING/OTUD5 protein in non-small cell lung cancer (NSCLC) tissues and corresponding noncancerous lung tissues. Scale bar, 500 μm. **b** Quantification of STING/OTUD5 IHC staining in noncancerous lung tissues ($n = 17$) and NSCLC tissues ($n = 73$). $^{**}p < 0.01$, multiple *t*-tests were performed to compare the samples. **c** Positive rate and correlation analysis of immunohistochemistry (IHC) staining with an anti-STING or an anti-OTUD5 Ab in noncancerous lung tissues ($n = 17$) and NSCLC tissues ($n = 73$). $^{**}p < 0.01$, simple linear regression. **d** Tumors were excised on day 3 after radiation and then were homogenized in PBS with protease inhibitor. After homogenization, Triton X-100 was added to obtain lysates. ELISAs were performed to measure IFN-β production. **e** Bioluminescent images and the corresponding quantification of tumors are shown for *Otud5*$^{fl/Y}$ and CD11-Cre *Otud5*$^{fl/Y}$ mice implanted with B16-Luc cells ($n = 8$ per group). Similar results were obtained in three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$

5′-GCATGAGGTTGTGCGAAAG-3′, and m*Otud5*: 5′-CCGGAATATCC ACTATAAT -3′. Scramble siRNAs were used as a negative control in all RNA interference experiments.

Conditional knockout mice. *Otud5*$^{fl/Y}$ mice were generated by the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and were provided by Dr Lingqiang Zhang, State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine. Approximately 1.1 kb of the genomic region covering exon 2 was removed via Cre/loxP excision. Deletion of exon 2 caused a frameshift that disrupted downstream protein domains. *Otud5*$^{fl/Y}$ mice were bred with Lyz2-Cre or CD11-Cre transgenic mice to produce myeloid cell or DC cell-conditional *Otud5* knockout mice. *Otud5*$^{fl/Y}$ mice used in our experiments were Cre-negative littermates of the *Otud5*$^{fl/Y}$ mice. All mice used were 8- to 10-week-old male mice. All animal experiments were undertaken in accordance with the NIH Guide for the Care and Use of Laboratory Animals and received the approval of the Scientific Investigation Board of School of Basic Medical Science, Shandong University, Jinan, Shandong Province, China.

Isolation of mouse macrophages. Peritoneal macrophages were harvested from mice 4 days after thioglycollate (BD), and the cells were cultured in DMEM supplemented with 5% FBS as previously described.[32]

Otud5-*KO RAW264.7 macrophages*. *Otud5*-KO RAW264.7 macrophages were generated with CRISPR-Cas9 methodology. A guide RNA was designed and cloned into a lenti-CRISPRv2 vector. The guide RNA sequence used was as follows: *Otud5* sg 5′-CAC CGGACCGTGACTCCGGCGTCGT-3′. The cells were cultured at 37 °C with 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Enzyme-linked immunosorbent assay (ELISA). The concentrations of IFN-β in culture supernatants and sera were measured by ELISA kits (BioLegend).

Coimmunoprecipitation (Co-IP) assay. For Co-IP, HEK293T cells transfected with the indicated constructs were lysed with IP buffer (10 mm Tris, pH 8.0, 150 mm NaCl, 1 mm EDTA, 1% NP-40, and 10% glycerol with a protease inhibitor cocktail, Sigma-Aldrich). The lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant was incubated with the indicated antibodies overnight at 4 °C, which was followed by 2 h of incubation at 4 °C with protein A or G-Sepharose beads. Then, after five washes with IP

buffer, the beads were eluted by boiling in sample buffer for SDS-PAGE, and immunoblot analysis was conducted with the indicated antibodies. The amounts of transfected plasmids were as follows: Myc-STING or its mutants 1.5 μg, Flag-OTUD5, or its mutants or other DUBs 2 μg, HA-Ub and its point mutants 1 μg.

In vitro deubiquitination assays. Flag-OTUD5 or C224S was expressed with a TNT Quick Coupled Transcription/Translation System (Promega) according to the instructions of the manufacturer. HEK293T cells were transfected with HA-Ub and Myc-STING. Forty-eight hours after transfection, cells were lysed with IP buffer containing protease inhibitors, and anti-Myc-conjugated beads were used to immunoprecipitate Myc-STING, and a large amount of Myc-STING was ubiquitinated. Immunoprecipitated complexes were washed and incubated with recombinant OTUD5 or C224S in deubiquitination assay buffer (50 mm HEPES/NaOH, pH 7.8, 0.5 mm EDTA, 1 mm DTT, and 0.1 mg/ml ovalbumin) at 37 °C for 1 h. The reaction was terminated by adding SDS sample buffer followed by 5 min of heat denaturation at 95 °C. The ubiquitination of STING was detected by immunoblotting with HA antibodies.

In vitro binding assay. Myc-STING and Flag-OTUD5 proteins were expressed with a TNT Quick Coupled Transcription/Translation System (Promega) according to the instructions of the manufacturer. Myc-STING and Flag-OTUD5 were mixed and then were immunoprecipitated before being subjected to immunoblots with the indicated antibodies.

Native polyacrylamide gel electrophoresis (PAGE). IRF3 dimerization assays were performed as described previously.[33] Gels were prerun with running buffer (25 mM Tris-Cl, pH 8.4, 192 mM glycine, in the presence or absence of 0.2% deoxycholate in the cathode and anode buffers, respectively) at 45 mA for 30 min. The lysate was centrifuged at 120,000 rpm for 5 min to remove the insoluble fraction. The sample was mixed with 2× loading buffer (125 mM Tris-Cl, pH 6.8, 30% glycerol, and 0.002% bromphenol blue) and was applied to the gel. The samples were electrophoresed at 25 mA for 50 min in a cold room.

Plaque assays and detection of virus replication. *Otud5*$^{fl/Y}$ or Lyz2-Cre *Otud5*$^{fl/Y}$ macrophages were infected with VSV (multiplicity of infection [MOI] = 0.1) or HSV-1 (MOI = 10) for the indicated times. HSV and VSV plaque assays were performed as described previously and replication rates were determined.[34]

Tumor samples and immunohistochemistry. Tumor samples for non-small cell lung cancer tissues and corresponding noncancerous lung tissues were purchased from Wuhan Sevier Company Ltd. (LAC-1402). Immunohistochemical detection of STING and OTUD5 proteins was performed on the chip. The tissue chip was scanned with Pannoramic MIDI (3D HISTECH). H-SCORE is the histochemistry score system, which is a histological scoring method for processing immunohistochemical results. The staining intensity was converted into corresponding values to achieve semiquantitative tissue staining. H-SCORE = $\sum$(PI × *I*) = (percentage of cells of weak intensity × 1) + (percentage of cells of moderate intensity × 2) + (percentage of cells of strong intensity × 3), where PI represents the number of positive cells out of the total number of cells in the section, and *I* represents the intensity of staining.

Quantification and statistical analysis

All data are presented as the mean ± SD of one representative experiment. Statistical significance was determined with one-way ANOVA, simple linear regression, and multiple *t*-tests, which were performed for each experiment, and a p-value < 0.05 was considered statistically significant.

DATA AVAILABILITY

All data generated or analyzed during this study are included in Figs. 1–6 and Supplementary Figs. S1–S5. Additional data sets that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

C.G. conceived and designed the research; Y.G., F.J., L.K., J.Z., B.C., and Y.L. performed the research; H.W., H.Z., and X.C. provided reagents; C.M., F.Y., L.Z., B.L., and Y.Z. provided discussions; L.Z. provided *Otud5*$^{fl/Y}$ mice; Y.G., F.J., and C.G. analyzed the data; and C.G., Y.Z., and Y.G. wrote the paper.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41423-020-00531-5) contains supplementary material.

Competing interests: The authors declare no competing interests.

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