STING Licensing of Type I Dendritic Cells Potentiates Antitumor Immunity

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Abstract: Stimulator of interferon genes (STING) is an immune adaptor protein that senses cyclic GMP-AMP (cGAMP) in response to self or microbial cytosolic DNA as a danger signal. STING is ubiquitously expressed in diverse cell populations including cancer cells with distinct cellular functions such as activation of type I interferons, autophagy induction, or triggering apoptosis. It is not well understood whether and which subsets of immune cells, stromal cells, or cancer cells are particularly important for STING-mediated antitumor immunity. Here using a polymeric STING-activating nanoparticle (PolySTING) with a shock-and-lock dual activation mechanism, we show type 1 conventional dendritic cell (cDC1) is essential for STING-mediated rejection of multiple established and metastatic murine tumors. STING status in the host but not in the cancer cells (*Tmem173*$^{-/-}$) is important for antitumor efficacy. Specific depletion of cDC1 (*Batf3*$^{-/-}$) or STING deficiency in cDC1 (*XCR1*^{cre}*STING*$^{fl/fl}$)

abolished PolySTING efficacy, whereas depletion of other myeloid cells had little effect. Adoptive transfer of wildtype cDC1 in *Batf3*$^{-/-}$ mice restored antitumor efficacy while transfer of cDC1 with STING or IRF3 deficiency failed to rescue. PolySTING induced a specific chemokine signature in wildtype but not *Batf3*$^{-/-}$ mice. Multiplexed immunohistochemistry analysis of STING-activating cDC1s in resected tumors correlates with patient survival while also showing increased expressions after neoadjuvant pembrolizumab therapy in non-small cell lung cancer patients. Therefore, we have defined that a subset of myeloid cells is essential for STING-mediated antitumor immunity with associated biomarkers for prognosis.

One Sentence Summary: A shock-and-lock STING-activating nanoparticle drives antitumor immunity through cDC1s with distinctive chemokine signatures

INTRODUCTION

STING (aka MITA, MYPS and encoded by *TMEM173* gene) is an endoplasmic reticulum (ER)-associated signaling protein that is essential for transcriptional regulation of numerous host defense genes against infection and cancer (*1-3*). STING is activated by 2', 3'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) (*4, 5*), an endogenous secondary messenger, which is produced by cGAMP synthase (cGAS) in response to microbial or self-DNA as a danger signal (*6*). Upon cGAMP binding, STING homodimer undergoes extensive conformational change that leads to oligomerization on the ER surface and translocation to the Golgi. STING oligomerization recruits and activates TANK-binding kinase 1 (TBK1) and inhibitor of κB kinases (IKKs), which in turn activate interferon regulatory factor 3 (IRF3) and nuclear factor κB (NFκB), respectively. IRF3 and NFκB cooperate to induce type I interferons and other inflammatory cytokines to mount a robust innate immune response. Besides immune activation, STING also triggers autophagy as a primordial function of host-defense independent of TBK1 and IKK (*7*).

Genomic instability of cancer cells places DNA sensing and cGAS-STING signaling as a key immune activation mechanism against cancer (*3*). However, immune system often displays dual host-

protective and tumor-promoting responses that are antipodally activated to modulate immunity (*8*). This dichotomous relationship is illustrated by contradicting reports where STING signaling has opposite effect on antitumor response. For immune protection, DNA from dying cancer cells was shown to activate cGAS-STING pathway, leading to the induction of type I interferons that prime T cells and natural killer cells against tumor cells (*9, 10*). On the other hand, chromosome instability has also been reported to activate the cGAS-STING pathway with inflammatory responses that promote cancer metastasis (*11, 12*). Massagué *et al* show cGAMP transfer from tumor cells to astrocytes produces inflammatory cytokines which activate STAT1 and NF-kB pathways leading to tumor growth and chemoresistance (*12*). More recently, they reported an opposite effect where STING signaling in cancer cells suppresses metastatic outbreak in an indolent lung cancer model (*13*). These findings illustrate that due to the broad expressions of STING in most host cells, multifaceted cellular functions likely exert complex functions in tumor control. Consequently, understanding the cellular context and spatio-temporal control of STING signaling is important to elucidate mechanisms that drive antitumor immunity.

In this study, we leveraged a polymeric STING-activating nanoparticle (PolySTING, Fig. 1A) to investigate the role of immune cell subset in STING-mediated antitumor immunity. Prior work using small molecule STING agonists (e.g., cGAMP, Adu-S100) have shown that they are readily taken up by T cells, leading to STING-mediated T cell deaths (*14, 15*) and T cell memory loss (*16*). Our previous work also showed STING activation by cGAMP alone led to rapid STING activation within 6 hours (shock) followed by lysosomal degradation and loss of function after 12 hours, whereas a synthetic polymer induced a slower but prolonged expressions of type I interferons over 48 hours (lock) through polymer-mediated STING biomolecular condensation (*17*). In the current PolySTING design, cGAMP is encapsulated within PSC7A micelles to create a 'shock-and-lock' STING nanoparticle agonist. This design synergizes the burst STING activation via cGAMP with non-canonical, sustained activity from PSC7A. PolySTING displays tropism toward myeloid cells with minimal uptake in T cells to avoid STING-mediated T cell deaths. The cell tropism and shock-and-lock STING activation by PolySTING allow the identification of conventional type 1 dendritic cells (cDC1s) as a key cell driver for the rejection

of malignant tumors. Our findings reveal direct targeting of cDC1s by a dual activating STING agonist offers a promising approach for STING-mediated cancer immunotherapy.

RESULTS

PolySTING is a potent STING agonist in hot and cold tumors

We produced PolySTING nanoparticles by encapsulating cGAMP in a micelle nanoparticle self-assembled from a STING-activating polymer, polyethylene oxide-*b*-PSC7A (PSC7A, Fig. 1A and fig. S1A). After cGAMP loading in PSC7A micelles, dynamic light scattering analysis shows that nanoparticles had a diameter of 25±3 nm, and a spherical morphology by transmission electron microscopy (fig. S1A). After incubation in ISG-THP1 cells over 24 hours, PolySTING produced robust IFN-β expression (>250-fold over saline control) compared to cGAMP (3-fold) or PSC7A NP (12-fold) groups (Fig. 1B). Time-dependent cytokine analysis illustrates a shock-and-lock mode of STING activation by PolySTING compared to a transient response by cGAMP (fig. S1B).

We used Cy5-labeled PolySTING to quantify the nanoparticle biodistribution after intratumoral administration in MC38 colon carcinoma. Abundant accumulation of PolySTING was found in the tumor and tumor-draining lymph nodes (tDLN) at 24 hours over other organs (Fig. 1C). Flow cytometry analysis showed significantly higher cell uptake in the immune cells ($CD45^+$) over cancer cells ($CD45^-$) in MC38 tumors (Fig. 1D). We identified significant tropism of PolySTING toward dendritic cells, macrophages, and natural killer cells over T cells in the tumor microenvironment and tDLNs. In contrast, FITC-labelled cGAMP did not show dramatic cell tropism across immune cell populations in tumor tissues, and only negligible cGAMP signal (<1%) was found in tDLNs (fig. S1C). *In vitro* studies show bone marrow-derived dendritic cells (BMDCs) or macrophages (BMDMs) readily endocytosed PolySTING with increased cGAMP uptake over free cGAMP (fig. S1, D to F). We next evaluated the effect of PolySTING (50 μg) versus Adu-S100 (50 μg), a small molecule STING agonist (*16*), on T cell viability and tumor growth inhibition. We observed increased apoptosis in $CD8^+$ T cells 24 hours after one injection of Adu-S100 compared to PolySTING (Fig. 1E and fig. S1G). Furthermore, MC38 tumors regressed after initial treatment by Adu-S100 followed by relapse, whereas PolySTING treatment led to complete tumor eradication (Fig. 1F). Additional dose-response studies show PolySTING achieved a

much broader therapeutic window from 20 to 100 μg for tumor eradication, whereas Adu-S100 had the optimal antitumor effect at 20 μg (2/5 tumor free) (fig. S1, H and I).

PolySTING displayed broad antitumor efficacy with prolonged survival compared to free cGAMP or glucose controls in immunogenic (e.g., MC38, human papilloma virus E6/E7-transfected TC-1 tumor, Fig. 2, A and B, and fig. S2, A and B) and immune-cold murine tumors (e.g., B16F10 melanoma, Lewis lung LLC2 carcinoma, Fig. 2, C and D, and fig. S2, C and D). To further explore if local treatment can generate sufficient T cells against metastasis, we selected triple negative 4T1 orthotopic mammary carcinoma that will have spontaneous lung metastasis starting on second week after inoculation. PolySTING monotherapy on day 11 not only inhibited the primary tumor growth, but also generated systemic immunity against the formation of metastatic 4T1 foci in the lung (Fig. 2E and fig. S2E). PolySTING treatment was also more potent than anti-PD-1 treatment for reducing metastasis (Fig. 2E). In MC38 and B16F10 distal tumor models, PolySTING also inhibited growth in distal tumors after injection in the primary tumors (Fig. 2F, and fig. S3, A to C). Furthermore, combination of PolySTING with anti-PD-1 resulted in therapeutic synergy in MC38 distal tumors. Mice cured by PolySTING displayed long-term T cell memory as shown by the rechallenge of MC38 cells at both the healed primary tumor location and the contralateral site (Fig. 2G and fig. S3D). Therefore, local treatment with PolySTING generates potent systemic immunity at distal and metastatic sites with induction of long-term memory.

Host STING-IFN-I signaling is important for PolySTING-mediated antitumor efficacy

We next investigated the role of STING in the host versus MC38 cancer cells in antitumor immunity. We compared tumor growth inhibition using STING wildtype MC38 cancer cells in host *Tmem173*$^{-/-}$ (STING knockout) mice, and *Tmem173*$^{-/-}$ MC38 cells in STING wildtype mice. PolySTING-mediated tumor eradiation was abolished in host *Tmem173*$^{-/-}$ animals with MC38 tumors but was retained in *Tmem173*$^{-/-}$ MC38 tumors with wildtype mice (Fig. 3, A to D). The predominant role of host STING in immune protection is also reported in an E0771 tumor model after ionizing radiation (*18*). Without PolySTING treatment, faster MC38 tumor growth was observed in *Tmem173*$^{-/-}$ mice than

wildtype mice. STING-mediated type I interferon (IFN-I), nuclear factor κB (NF-κB), and/or autophagy signaling are proposed to contribute to host defense against cancer or viral infection (*7, 10, 19, 20*). We evaluated the efficacy of PolySTING in mice deficient of the interferon-α/β receptor (*Ifnα/βR*$^{-/-}$), and a STING mutant mouse (STING^{S365A}) that blocks IRF3 binding and IFN-I production but not NF-κB activation or autophagy induction (*20*). Neither animal models exhibited antitumor efficacy upon PolySTING treatment over the wildtype mice (Fig. 3, E and F). Collectively, these data indicate that the host STING-IFN-I signaling is essential in PolySTING-mediated immunity against MC38 tumors.

cDC1 drives STING-mediated tumor rejection

To determine which immune cell subsets are important for PolySTING-mediated effects, we used antibody depletion or mice with deficiency in different subsets of antigen presenting cells. MC38 tumor growth inhibition was evaluated by antibody blockade of B cells (a mixture of anti-CD19/CD22/B220), macrophages (anti-CSF1R), and in transgenic mice with depletion of different DC subtypes, including monocyte-derived DC (MoDC, *Ccr2*$^{-/-}$) (*21*), plasmacytoid DC (pDC, anti-CD317) (*22*), cDC1 (*Batf3*$^{-/-}$) (*23*), and cDC2 (*Irf4*$^{-/-}$) (*24*). Depletions of B cells, macrophages, MoDCs, pDCs, and cDC2s did not exhibit a clear difference in antitumor efficacy over control mice after PolySTING treatment (Fig. 3, G to K). In contrast, mice with cDC1 deficiency (*Batf3*$^{-/-}$) abolished the antitumor efficacy by PolySTING (Fig. 3L). Although free cGAMP did not display obvious cell tropism (fig. S1C), it still required cDC1 to induce antitumor immunity, as tumor growth inhibition was also abrogated in *Batf3*$^{-/-}$ mice (fig. S4A). Compared to free cGAMP, PolySTING has a stronger therapeutic effect in wildtype mice, likely due to the higher and more persistent STING signaling (Fig. 1B and fig. S1B) and increased uptake in myeloid cells (fig. S1, D to F).

To further determine whether the therapeutic contribution of cDC1 is dependent on endogenous STING activation, or secondary to the IFN-I produced by neighboring cells, we constructed and investigated mice with conditional knockout of STING in cDC1 (*XCR1*^{cre}*STING*$^{fl/fl}$, fig. S4B) and monitored tumor growth after PolySTING treatment (Fig. 4A and fig. S4C). Tumor

growth inhibition of MC38 tumors by PolySTING was abrogated in *XCR1*^{cre}*STING*$^{fl/fl}$ mice, indicating STING signaling within cDC1s is required for antitumor immunity.

Next, we performed cell transfer studies in *Batf3*$^{-/-}$ mice to evaluate the sufficiency of cDC1 in STING-mediated antitumor efficacy. BMDCs were treated with GM-CSF, IL-4 and Flt3-L, and cDC1s were isolated and enriched through flow sorting (Fig. 4B). Combined CD8α^{+} and CD103^{+} cDC1s were subcutaneously injected adjacent to the MC38 tumors 12 hours prior to PolySTING treatment. Notably, adoptive transfer of cDC1s restored antitumor immunity and prolonged survival by PolySTING in *Batf3*$^{-/-}$ mice, whereas PolySTING treatment or cDC1 transfer alone showed negligible efficacy (Fig. 4C, and fig. S5, A and B). Transfer of cDC1s from STING knockout or STING^{S365A} mutant mice did not lead to immune protections against MC38 tumors in *Batf3*$^{-/-}$ mice (Fig. 4D). Lack of immune protection by cDC1 transfer from STING^{S365A} mutant mice supports previous findings that type I interferon is required by dendritic cells for immune rejection of tumors (*25, 26*).

To better define tumor specific responses, we utilized the MC38-OVA tumor model to investigate the priming of transferred cDC1s (wildtype versus STING knockout) in *Batf3*$^{-/-}$ mice (Fig. 4E). In the MC38-OVA tumors, a significant increase of both subsets of wildtype cDC1s was observed with 10- and 4-fold higher CD8α^{+} and CD103^{+} cDC1s in PolySTING group over the glucose control, respectively. In contrast, STING deficient cDC1s show marginal difference between the two groups. We also observed an increase in OVA peptide (SIINFEKL) presentation on H-2K^{b} MHC-I complex for wildtype cDC1s, where PolySTING treatment elevated antigen presentation over 15- and 4-fold for CD8α^{+} and CD103^{+} cDC1s, respectively. In contrast, STING deficient cDC1s did not show any significant changes after PolySTING treatment in either subset of cDC1s. Co-stimulation of cDC1s (e.g., CD86) was also elevated in both subsets of cDC1s after PolySTING treatment. These results illustrate robust STING-dependent cDC1 infiltration and activation after PolySTING treatment.

PolySTING boosts cDC1 activation and priming of CD8^{+} T cells *in vivo*

We further investigated STING-mediated potentiation of dendritic cells in antigen presentation, co-stimulation and $CD8^+$ T cell priming after intratumoral injection in MC38-OVA tumors (fig. S6A). Notably, a dose of 50 μg of PolySTING induced the strongest cDC1 activation level and cytotoxic T cell response compared to 10 or 200 μg doses in both tumors and tDLNs (fig. S6, B to F), in accordance with prior therapeutic efficacy findings (fig. S1I).

We next performed a side-by-side comparison of PolySTING (50 μg) and Adu-S100 (50 μg) (Fig. 5A). We observed increased cDC1s in the MC38-OVA tumors from PolySTING group compared with Adu-S100 (2.4-fold) or glucose control (7.1-fold) (Fig. 5B). Co-stimulation signals (e.g., CD80) were dramatically increased in both the $CD8\alpha^+$ and $CD103^+$ cDC1s over Adu-S100 group (>15-fold, Fig. 5C). The percentage of dendritic cells with OVA peptide (SIINFEKL) presentation on $H\text{-}2K^b$ MHC-I complex also significantly increased in the PolySTING group over the glucose control, but the difference with Adu-S100 group was not significant (Fig. 5D). Dendritic cell activation by PolySTING resulted in significantly increased production of OVA-specific $CD8^+$ T cells in both the tumor microenvironment and tumor-draining lymph nodes than the Adu-S100 and glucose groups (Fig. 5E). Tumor-resident $CD8^+$ T cells were cytotoxic, as demonstrated by the elevated interferon-γ and granzyme B expressions from PolySTING treatment (Fig. 5F). Combined with the previous data on Adu-S100-induced apoptosis in $CD8^+$ T cells (Fig. 1E), these results highlight the importance of cell selectivity by PolySTING in mounting a productive antitumor immunity over known STING agonists such as Adu-S100.

$CD103^+$ cDC1s from MC38 tumors also exhibited significantly increased expression of X-C motif chemokine receptor 1 (XCR1), C-type lectin domain containing 9A (CLEC9A) and C-C motif receptor 7 (CCR7) after STING activation (Fig. 6A). CCR7 is a cell surface chemokine receptor that drives DC homing to lymphoid organs (*27*). In the tumor draining lymph nodes, elevated CCR7 expressions were found on both subsets of cDC1s (Fig. 6B). *CCR7*$^{-/-}$ mice rejected cDC1 migration to lymph nodes and lost STING-mediated tumor growth inhibition (Fig. 6, C and D). These data demonstrate cDC1 trafficking to tumor-draining lymph nodes is important to prime the tumor-specific T cell immunity.

Next, we depleted NK, $CD4^+$ or $CD8^+$ T cells and investigated their impact on PolySTING-mediated tumor clearance. Data revealed an indispensable role of $CD8^+$ T cells but not NK or $CD4^+$ T cells in tumor rejection (Fig. 6, E to G). Through an *in vivo* priming assay, MC38 tetramer$^+$ $CD8^+$ T cells yielded approximately 5-fold higher proliferation in the PolySTING treatment group over control in wildtype C57BL/6 mice (Fig. 6H). In contrast, only minimal tetramer$^+$ $CD8^+$ T populations were detected in *Batf3*$^{-/-}$, *Tmem173*$^{-/-}$, *Ifnα/βR*$^{-/-}$, *or STING*^{S365A} mice, whereas *Irf4*$^{-/-}$ (cDC2 knockout) mice maintained the production of antigen-specific $CD8^+$ T cells. In summary, these data illustrate the important role of cDC1 activation by PolySTING in antigen presentation, co-stimulation, and migration to lymph nodes for the priming of $CD8^+$ T cells against tumor cells.

PolySTING-activated chemokine signature is associated with improved patient survival

We next quantified the immune profiles in MC38 tumors from wildtype and cDC1 deficient (*Batf3*$^{-/-}$) mice after STING stimulation. CXCL9/10 and C-C motif chemokine ligand 4 and 5 (CCL4/5) were the top upregulated chemokines (>10-fold) in wildtype mice after PolySTING treatment (Fig. 7A and fig. S7). In *Batf3*$^{-/-}$ mice, significantly reduced expression levels of CXCL9/10 and CCL4/5 were observed after PolySTING treatment, whereas such differences were not detected in Adu-S100 groups (Fig. 7B). Interestingly, Adu-S100 presented a different chemokine profile, wherein CXCL9 was downregulated, while CCL20 and CXCL1 were upregulated (fig. S7A).

Chemokines CCL5 and X-C motif chemokine ligand 1 (XCL1) from NK cells have been reported to induce cDC1 migration into tumors (*28*). RT-PCR analysis of MC38 tumors showed a significant increase in mRNA expression of CCL4 and CCL5, but not XCL1, after PolySTING treatment (fig. S8A). An *in vitro* chemotaxis assay showed significant cDC1 enrichment in transwells containing CCL4 or CCL5, confirming their induction effect in cDC1 migration (fig. S8B). In mice, PolySTING expanded the infiltration of both subsets of cDC1 cells in the tumor microenvironment, while neutralization of CCL4/5 retarded STING-mediated cDC1 migration (fig. S8C) and tumor growth inhibition and survival (fig. S8, D and E).

We next examined The Cancer Genome Atlas (TCGA) database to evaluate PolySTING-induced

chemokine signatures (CCL4/5, CXCL9/10) in cancer patients with skin cutaneous melanoma or sarcoma. In parallel, we also probed BATF3, CLEC9A, XCR1 and CLNK, a collective of biomarkers reported for cDC1s (*28*). Positive correlations were found between STING chemokine and cDC1 signatures in both sets of patient samples (Pearson correlation coefficient $r>0.7$, fig. S9, A and B). Furthermore, PolySTING-induced chemokine signatures (CCL4/5, CXCL9/10) were associated with improved survival outcomes in both cancer patient populations, whereas analysis by bulk STING expressions has no statistical significance (fig. S9, A and B.)

STING-cDC1 signature is elevated in resected patient tumors after neoadjuvant immunotherapy

Based on the mechanistic findings from our murine studies, we next performed multiplex immunohistochemistry (mIHC) staining of formalin-fixed and paraffin-embedded tumor tissues from non-small cell lung cancer patients who have received either neoadjuvant chemotherapy (NAC, n=30) or neoadjuvant Pembrolizumab and chemotherapy (NAPC, n=28) (Table S1). We used $XCR1^{+}STING^{+}CXCL9^{+}$ biomarkers to stain for STING-activating cDC1 cells, and further investigated their physical contact with $CD8^{+}$ T cells in the tumor microenvironment (Fig. 7C and fig. S9C). Patients who received NAPC therapy significantly elevated STING-activating cDC1 signatures over patients treated by chemotherapy alone ($p<0.001$, Fig. 7D). The activated cDC1 further engages $CD8^{+}$ T cells to augment the antitumor immunity. In accordance with mIHC analysis, we further divided the cancer patients into STING-activating $cDC1^{high}$ (above medium level) and $cDC1^{low}$ (below medium level) groups. STING-activating $cDC1^{high}$ postulated a positive prediction of prolonged progression free survival and overall survival in both NAC and NAPC patients, whereas low abundance of STING-activating cDC1 signature led to poor survival outcome more pronouncedly in patients without immunotherapy (Fig. 7E and fig. S9D). These findings indicate that the STING-activating cDC1 signature ($XCR1^{+}STING^{+}CXCL9^{+}$) may serve as a prognostic clinical biomarker for cancer patient response to therapies.

DISCUSSION

STING is a confluent immune signaling protein that plays an important role in immune defense against malignancy. However, so far therapeutic targeting of STING has only met with moderate clinical response. Intratumorally injected Adu-S100 has shown 2% partial response, 38% stable disease and 60% progressive disease in a Phase I clinical study (*29*). Our cell uptake study of a similar small molecule STING agonist (cGAMP-FITC) showed the lack of strong cell selectivity across different immune cell subsets, where the molecule was readily taken up by the T cells (fig. S1C). This result is in accordance with previous reports where STING activation in T cells induced cell deaths (*14, 15*), and was further validated in our MC38 tumor model (Fig. 1E). To overcome this limitation, we utilized PolySTING, a nanoparticle STING agonist, to selectively target myeloid cells but not T cells, thereby preventing T cell deaths (Fig. 1, D and E). Compared to cGAMP where only transient IFN-β expressions were observed, PolySTING produced a shock-and-lock mode of STING activation with increased intensity and duration of STING signaling (fig. S1B). Dose-response studies illustrate PolySTING achieved a significantly broader therapeutic window with robust tumor rejection in a 5-fold dose range, whereas Adu-S100 had an optimal response at 20 μg per mouse with quick degradation of efficacy at other doses (fig. S1, H and I). Immune profile analysis showed different chemokine and cytokine signatures despite both therapies target the STING pathway. For example, PolySTING treatment increased the expressions of CCL4/5 and CXCL9/10 whereas Adu-S100 elevated CCL20 and CXCL1. CXCL9/10 produced by cDC1s have been reported to drive adoptive T cell infiltration into tumors (*30*), and CCL4/5 upregulation in ovarian cancer patients has shown favorable response to checkpoint blockade therapy (*31*). In contrast, CCL20 and CXCL1 have been reported to increase the infiltration of Tregs and MDSCs, which tempers the inflamed tumor microenvironment (*32*). These data indicate spatio-temporal control of STING signaling in selected immune cell populations are critical in controlling antitumor immunity.

Nanoparticle STING agonists may hold therapeutic advantages over small molecule STING agonists (*33-35*). Irvine *et al* showed lipid nanodiscs increased dendritic cell infiltration in the MC38 tumors for T cell priming (*33*). Weichselbaum *et al* reported zinc cyclic di-AMP nanoparticles disrupted

tumor endothelial cells and targeted macrophages for augmented antitumor immunity (*35*), whereas Wilson *et al* showed STING-activation nanoparticles led to vascular normalization with improved vascular integrity and T cell infiltration (*36*). In this study, using PolySTING and MC38 tumors as a model system, we systematically investigated the contributions of different immune cell subsets in tumor clearance. Data show depletion of cDC2, pDC, moDC, macrophages in the myeloid compartment or blocking of B cells had minimal effect on tumor rejection (Fig. 3, G to K), whereas mice deficient of cDC1s (*Batf3*$^{-/-}$) abrogated antitumor efficacy of PolySTING (Fig. 3L). This cDC1 requirement is corroborated by a recent study using cGAMP-loaded virus-like nanoparticles (*37*). Our data further demonstrate STING signaling within cDC1s is important because PolySTING efficacy is abolished in *XCR1*^{cre}*STING*$^{fl/fl}$ mice (Fig. 4A), as well as transfer of cDC1s from STING knockout mice cannot rescue the abrogated PolySTING efficacy in *Batf3*$^{-/-}$ mice (Fig. 4D). Results from STING^{S365A} mutant mice further reveal the importance of IRF3 signaling downstream of STING in cDC1 activation by PolySTING.

Based on the mouse findings, we introduced a STING-cDC1 biomarker (XCR1-STING-CXCL9) to evaluate the resected tumors from non-small cell lung cancer patients treated with Pembrolizumab in combination with chemotherapy (Fig 7, C and D, and fig. S9C). Compared to chemotherapy alone, Pembrolizumab treatment elevated the STING-cDC1 signatures, illustrating checkpoint blockade therapy can impact cDC1 recruitment and activation in the tumor microenvironment in a subset of patients. The activated cDC1s may further invigorate the CD8^{+} T cells to augment antitumor immunity. The correlation between STING-cDC1^{high} and improved progression-free survival and overall survival were demonstrated (Fig. 7E and fig. S9D). For cancer patients who have low STING-cDC1 signatures even after immune checkpoint therapy (e.g., ~50% patients in Fig. 7D), PolySTING may offer a useful therapeutic strategy to directly boost cDC1 infiltration and activation.

Despite the therapeutic promise, the current study did not fully address many potential hurdles when translating the STING nanoparticles into the clinics. First, compared to small molecule drugs, nanotherapeutics such as PolySTING are more complex in structure and thus are difficult to scale up while maintaining batch-to-batch reproducibility. The added costs and steps in chemistry, manufacturing,

and controls (CMC) may be daunting. Second, although PolySTING (and other nanoparticles including VLPs) therapies present a boosted therapeutic outcomes and immune profiles over small molecule drugs, they may also heighten the immune-related toxicity and autoimmunity. Finally, it remains to be seen whether safety and antitumor efficacy findings in mice can be recapitulated in human patients that have highly different host and cancer genetics and history of disease progression.

In summary, using a nanoparticle STING agonist, we discovered the importance of STING licensing of conventional type I dendritic cells (cDC1s) over other immune cell subsets in mounting a robust antitumor immunity. A STING-activating cDC1 signature may serve as a prognostic biomarker for patient response to cancer immunotherapy. Our findings indicate direct targeting of STING pathways in cDC1s may offer an effective strategy to restore protective immunity against solid tumors.

MATERIALS AND METHODS

Study design

The primary objective of this study was to use a "shock-and-lock" STING-activating nanoparticle (PolySTING) as a tool to uncover new biological insights on cell context dependence of STING-mediated antitumor immunity. Conventional small molecule agonists (2'3'-cGAMP or Adu-S100) were used as a treatment of reference. The tumor growth and animal survival of different tumor-bearing mice were monitored. Mice of specific gene knockout or conditional knockout were genotyped, while mice of specific immune cell blockade were confirmed via flow cytometry before each experiment. Through loss (*XCR1*^{cre}*STING*$^{fl/fl}$)- and gain (cDC1 adoptive transfer)-of-function studies, we demonstrated direct STING signaling in cDC1 is both necessary and sufficient for the rejection of multiple established and metastatic murine tumors. The immune orchestration of cDC1 and CD8^{+} T cells in tumors/tDLNs by different treatments was carefully characterized and the chemokine/cytokine profiles of tumors were quantified in wildtype and cDC1-deficient mice. We further collected tumor tissues from non-small cell lung cancer patients who have received NAC or NAPC treatment, and performed mIHC staining to evaluate the clinical value of STING-activating cDC1 signature. Sample size was determined via a priori power analysis based on means and SEMs estimated from pilot experiments or the literature. The number of samples combined, and the number of independent experiments is included in the figure legends.

Patient participants and ethical approvals

A total of 58 non-small cell lung cancer (NSCLC) patients who received two cycles of neoadjuvant chemotherapy (NAC, 30 cases) or neoadjuvant pembrolizumab chemotherapy (NAPC, 28 cases) prior to surgical resection were included in this study. The squamous cell carcinoma patients received neoadjuvant paclitaxel 175 mg/m^2 plus carboplatin (area under curve 5; 5 mg/mL per min) and the

adenocarcinoma patients received pemetrexed 500 mg/m^2 plus carboplatin, with or without intravenous pembrolizumab 200 mg on day 1, 21 days each cycle, for two cycles before surgical resection, and then followed by two cycles after surgical resection. Clinicopathological parameters included age, gender, smoking index, clinical tumor node metastasis (TNM) stage, pathological type. Progression-free survival (PFS) was defined as the time from the date of surgery to the date of recurrence or last follow-up. Overall survival (OS) was defined as time from the date of surgery to the date of any caused death or last follow-up. The last follow-up was conducted on October 10, 2022, with a median follow-up time of 97 and 35 months for NAC and NAPC patients, respectively. The study was approved by the Ethics Committee of Tianjin Cancer Institute & Hospital (protocol number: bc2020060). All patients enrolled in the study provided written informed consent.

Cell lines and culture

Tmem173$^{-/-}$ MC38 cells were generated by knocking out *Tmem173* genes by CRISPR/Cas9 technology following a published procedure (*38*). The guide sequence 5'-CACCTAGCCTCGCACGAACT-3' for *Tmem173* was cloned into px458 plasmids (non-integrating plasmid with GFP selecting marker), and then were transiently transfected into tumor cells using lipofectamine 2000 (Thermo Fisher). LLC2 cells were kindly provided by Zhijian 'James' Chen, UT Southwestern Medical Center. TC-1 cells were kindly provided by T. C. Wu, Johns Hopkins University. 4T1-luciferase cells were kindly provided by S. Huang, Massey Cancer Center, Virginia Commonwealth University. MC38, MC38-OVA, and B16F10 cells were purchased from ATCC (Manassas, VA). All the cells were cultured in complete DMEM medium (Gibco-BRL Life Technologies, Canada) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco-BRL Life Technologies, Canada). THP1-ISG cells from ATCC were cultured in RPMI medium supplemented with 10% FBS and 0.05 mM β-mercaptoethanol (β-ME). All cells were grown at 37 °C in a humid atmosphere with 5% CO_2. Bone marrow cells were filtered through a 70 μm strainer and treated with Red Blood Cell Lysis Buffer (Sigma). Single-cell suspensions of bone marrow cells were cultured in RPMI-1640 medium containing 10% FBS, supplemented with 20 ng/ml GM-CSF and 10 ng/ml IL-4 for BMDC, or 20 ng/ml M-CSF for BMDM (Peprotech). The medium was half replenished every 2 days. Cell lines were routinely monitored for mycoplasma contamination. All cells used in this study tested negative for mycoplasma contamination.

Mice

XCR1^{cre} and *STING*$^{fl/fl}$ mice were purchased from the Jackson Laboratory. The *STING*$^{fl/fl}$ mice were first crossed with *XCR1*^{cre} mice to generate F1 mice. Then the F1 mice were inbred to generate the cre$^+$ mice with homozygous *STING*$^{fl/fl}$. The insertion of cre and flox elements were confirmed by PCR per Jackson Laboratory's protocol with the following primers: XCR1: TCAAGTTCCGCAGACACCTA, GTGCACGAAGTGTTGCTTTG, ACCGTCGACCCCATAGTCAT; STING: TTTTCATCTGCCTTCCAGGT, GCGCACACACACTAAAAACTG. *Tmem173*$^{-/-}$ mice, *IfnαβR*$^{-/-}$ mice, *Ccr2*$^{-/-}$ mice, *Batf3*$^{-/-}$ mice, *Irf4*$^{-/-}$ mice, *Ccr7*$^{-/-}$ mice were purchased from the Jackson Laboratory. *STING*^{S365A} mutant mice was generated by N. Yan's lab at UT Southwestern (*15*). C57BL/6 wild-type (*WT*) mice and BALB/c mice were purchased from Charles River Laboratories. All the mice were

maintained under specific pathogen-free conditions and housed in a barrier facility under a 12 h–12 h light–dark cycle and maintained on standard chow (2916, Teklad Global). The temperature range for the housing room is 68–79 °F (average is around 72 °F) and the humidity range is 30–50% (average is around 50%). The experimental groups included randomly chosen female littermates of ages around 8 weeks and the same strain. All experiments were performed in accordance with the procedures approved by the AAALAC-accredited Institutional Animal Care and Use Committee at UT Southwestern Medical Center and were compliant with all relevant ethical guidelines.

Preparation of PolySTING nanoparticles

PEO-*b*-P(MAC-SC7A·HCl) (PSC7A) copolymer was synthesized following a previously published procedure (*39*). cGAMP-PSC7A nanoparticles (PolySTING) were prepared by mixing 25 μL 2′3′-cGAMP (2 mg/mL) in 1 mL PSC7A polymer solution (1 mg/mL) containing 5% D-glucose at pH 5, and then adjusted to pH 7 using NaOH (1 M) to induce micelle assembly with cGAMP loading. After micelle formation, the nanoparticles were characterized using dynamic light scattering (Zeta Sizer, Malvern, He-Ne laser, λ = 633 nm) to measure the hydrodynamic size, size distribution and zeta potential. Transmission electron microscopy was used to analyze particle morphology. The cGAMP loading efficiency (>90%) was quantified via high-performance liquid chromatography (1260 Infinity II, Agilent).

Microscopic imaging of cell uptake of PolySTING

BMDCs and BMDMs were grown in a four-well glass chamber and treated with fluorescently labelled PolySTING for the indicated time. The cGAMP-FITC used in the experiment was purchased from BIOLOG Life Science Institute and encapsulated in Cy5-labeled PSC7A micelles. Cells were fixed in 4% paraformaldehyde, then stained for CD11c (Alexa Fluor 594 anti-mouse CD11c antibody, clone: N418, BioLegend), or CD11b (Alexa Fluor 594 anti-mouse CD11b antibody, clone: M1/70, BioLegend) using an immunofluorescence kit (Cell Signaling Technologies). Samples were stained with DAPI (Thermo Fisher Scientific) and imaged using the built-in software (ZEN 2.6) of the Zeiss 700 confocal laser scanning microscope with a 63× oil-immersion objective. ImageJ v.1.52d was used to quantify colocalization using the Pearson's correlation coefficient. Data are representative of at least 20 cells.

Tumor growth and treatments

For subcutaneous tumor xenografts, C57BL/6 mice were inoculated with 1×10^6 MC38 colon carcinoma cells, 1×10^6 LLC2 lung cancer cells, 1.5×10^5 TC-1 tumor cells, or 1.5×10^5 B16F10 melanoma cells on the right flank on day 0. The metastatic breast cancer model in Balb/c mice was constructed by mammary fat pad injection of $1x10^6$ 4T1-luciferase cancer cells. MC38 tumor-bearing C57BL/6 mice were treated by intratumoral injection of PolySTING (50 μL per mice) Adu-S100, or 5% glucose control on day 11, 14 and 17. B16F10 tumor-bearing mice were treated on day 8, 11 and 14. TC-1 tumor-bearing mice were treated on day 7, 10 and 13. LLC2 tumor bearing mice were treated on day 6, 9 and 12. 4T1 tumor-bearing mice were treated with PolySTING on day 11, 14 and 17. Meanwhile, anti-PD-1 (200 μg) was intraperitoneally injected one day before PolySTING and was given 3 times. Mice were monitored for tumor growth. Tumor volumes were measured with a caliper by the length (L), width (W) and calculated

as V = L×W×W/2.

For distal tumor model, C57BL/6 mice were subcutaneously injected with $1x10^6$ MC38 cells or $2x10^5$ B16F10 cells on the primary site, followed by subcutaneous injection of $1x10^6$ MC38 cells on day 4 or $2x10^5$ B16F10 cells on day 6 on the contralateral sites. Mice were intratumorally injected with PolySTING on day 10, 13 and 16. Anti-PD-1 was given one day before PolySTING. Mice were monitored for tumor growth on both primary and distal sites. For MC38 rechallenge study, the tumors of C57BL/6 wildtype mice were removed by surgical resection on day 30 and then mice were injected with $1x10^6$ MC38 cells on distal site on day 45. Meanwhile, C57BL/6 naïve mice were injected with MC38 cells on day 45. Cured mice from PolySTING treatment were rechallenged with the same amount of MC38 cells on primary site and distal site. Mice were monitored for tumor growth.

In *vivo* immune cell depletion experiments

For the NK cell depletion assay, mice were i.p. injected with anti-NK1.1 antibody (500 μg, clone PK136, BioXcell) on day 3 after tumor inoculation and then were maintained with 250 μg of anti-NK1.1 antibody every 3 days to deplete NK cells (from 10.5% to 2.4%). For $CD8^+$ or $CD4^+$ T cell depletion, mice were treated with anti-CD8a antibody (200 μg, clone YTS169.4, BioXcell) or anti-CD4 antibody (200 μg, clone GK1.5, BioXcell) by i.p. injection on day 3 after tumor inoculation and then were maintained with same dosage of antibodies every 3 days (from 10.3% to 0.19% for $CD8^+$ T depletion and from 17.8% to 0.73% for $CD4^+$ T depletion). For B cell depletion, mice were i.p. treated with a cocktail of antibodies (*40*), including CD19 (clone 1D3, BioXcell), B220 (clone RA3.3A1/6.1, BioXcell), and CD22 (clone CY34.1; BioXcell) weekly, each at 150 μg per mouse. After 48 h, the mice were injected with a secondary antibody (mouse anti-rat Kappa light chain, clone MAR 18.5, BioXcell) at 150 μg. In addition, mice were injected with anti-CD20 antibody (clone AISB12, BioXcell) at 250 μg weekly for 4 weeks (from 17.2% to 1.4%). For macrophage depletion, mice were treated with 500 μg of anti-CD115 antibody (clone AFS98, BioXcell) every 3 days (from 2.2% to 0.44%). Plasmacytoid dendritic cells (pDC) depletion in C57BL/6 mice was achieved by i.p. injection of mice with 200 μL of anti-PDCA1 antibody (clone 927, BioXcell) at 2 mg/mL (from 1.5% to 0.33%).

Adoptive transfer of dendritic cells

BMDCs were generated by culturing bone marrow cells flushed from the femurs of C57BL/6 naïve mice, STING knockout mice, or $STING^{S365A}$ mutant mice. The RPMI 1640 medium supplemented with 10% FBS, Pen/Strep, sodium pyruvate, 20 ng/mL GM-CSF, 10 ng/mL IL-4 and 200 ng/mL Flt-3L was prepared for culturing BMDCs. Non-adherent and loosely adherent immature DCs were collected on day 6 and induction efficacy of BMDCs was detected by determining the expression of CD11c (routinely 70%–80% $CD11c^+$). $CD103^+$ cDC1 and $CD8\alpha^+$ cDC1 were sorted by flow cytometry (*41*) and then mixed as total cDC1. Then $5x10^5$ cDC1s were subcutaneously injected into MC38 tumor-bearing *Batf3*$^{-/-}$ mice by the tumor one day before PolySTING treatment. Mice were monitored for tumor growth.

Flow cytometry

When tumor reached 150-200 mm^3 in size, two doses of PolySTING or other indicated control group

were injected intratumorally twice, and tumors/tDLNs were harvested one day after last injection for DC analysis and three days after for T cell analysis, respectively. Tissues were then digested using 160 μg/mL collagenase IV (Sigma–Aldrich) and 20 μg/mL DNase I (Sigma–Aldrich) in RPMI 1640 media at 37 °C for 30 minutes and then washed and strained through a 70 μm filter (BD Falcon). LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen™, L34966), PerCP anti-mouse CD45 (clone 30-F11, BD), Brilliant Violet 650 anti-mouse Ly-6C Antibody (clone HK1.4, BioLegend), Brilliant Violet 421 anti-Mouse CD11b (clone M1/70, BD), Brilliant Violet 786 anti-mouse CD11c (clone HL3, BD), PE/Cyanine7 anti-mouse I-A/I-E (clone M5/114.15.2, BioLegend), BV605 anti-mouse CD103 (clone M290, BD), Alexa Fluor 700 anti-mouse CD8a (clone 53-6.7, BioLegend), APC anti-mouse CD86 (clone GL-1, BioLegend), PE/Cyanine 7 anti-mouse CD80 (clone 16-10A1, BioLegend), PE anti-mouse XCR1 (clone ZET, BioLegend), PE anti-mouse CLEC9A (clone 7H11, BioLegend), PE/Cyanine 7 anti-mouse CCR7 (clone 4B12, BioLegend), Brilliant Violet 786 anti-mouse CD3e (clone 145-2C11, BD), Alexa Fluor 700 anti-mouse CD8 (clone 53-6.7, BioLegend), Brilliant Violet 605 anti-mouse CD4 (clone RM4-5, BioLegend), APC anti-mouse IFN-γ (clone XMG1.2, BioLegend), FITC anti-mouse Granzyme B (clone GB11, BioLegend), PE anti-mouse H-2K^b bound to SIINFEKL (clone 25-D1.16, BioLegend) APC anti-mouse T-Select H-2Kb MuLV p15E Tetramer-KSPWFTTL (code TB-M507-2, MBL) were used for flow cytometry analyses. Cell suspensions were incubated with Fc Block TruStain FcX Clone 93 (BioLegend) in PBS containing 0.5% bovine serum albumin and 2 mM EDTA before staining with fluorochrome labeled antibodies, followed by staining with live/dead dyes and selective antibodies of cell surface markers. Intracellular markers, including IFN-γ and granzyme B, were stained after cell fixation and permeabilization with True-Nuclear™ transcription factor buffer set per protocol (BioLegend). Data were collected on BD LSR Fortessa or Beckman CytoFLEX flow cytometer and analyzed by FlowJo (Tree Star Inc., Ashland, OR) software. All gating strategies were shown in fig. S10.

Tetramer$^+$ CD8$^+$ T cell assay

C57BL/6 wildtype mice as well as different types of genetic knockout mice (*Batf3$^{-/-}$, Tmem173$^{-/-}$, IfnαβR$^{-/-}$, STING^{S365A}, Irf4$^{-/-}$*) were subcutaneously inoculated with 1x10^6 MC38 cancer cells on the right flank on day 0. Mice were then treated with PolySTING when the tumor volume reached 150-200 mm^3. Tumor draining lymph nodes from these mice were made into single cell suspensions the second day after two injections of PolySTING. The ratio of tetramer$^+$ CD8$^+$ T cells with or without PolySTING treatment from mice were analyzed by flow cytometry.

Multiplex cytokine and chemokine analysis

MC38 tumor tissues from wildtype or *Batf3$^{-/-}$* C57BL/6 mice were harvested 12 hours after a single intratumoral injection of PolySTING, Adu-S100, or 5% glucose control. Tumor tissue homogenate preparation was performed and then analyzed for levels of cytokine/chemokine expressions by choosing mouse cytokine/chemokine 44-plex discovery assay array (MD44) according to the manufacturer's instructions (www.evetechnologies.com).

Tissue isolation and quantitative PCR analysis

Fresh MC38 tumor tissues were homogenized by using Precellys Evolution machine (Bertin). Total

RNAs were extracted from tumor tissues using the RNeasy mini kit (Qiagen). RNA quantity and quality was then confirmed using the NanoDrop (DeNovix DS-11) system. Genomic DNA was removed, and cDNA was synthesized using an iScript gDNA clear cDNA synthesis kit (Bio-Rad). Bio-Rad SsoAdvanced universal SYBR green supermix and CFX connect real-time system were used for PCR analysis. Results were corrected by GAPDH and plotted in GraphPad Prism version 9.0. The DNA primers used were as follows: mouse Ccl4: AGCCAGCTGTGGTATTCCTG, AGGGGCAGGAAATCTGAACG; mouse Ccl5: CTCACCATATGGCTCGGACA, CTTCTCTGGGTTGGCACACA; mouse Xcl1: CTTTCCTGGGAGTCTGCTGC, CAGCCGCTGGGTTTGTAAGT.

Dendritic cell chemotaxis assay

Chemotaxis of cDC1s was analyzed by using transwell migration assays. BMDCs ($1x10^6$ cells) were dispersed in RPMI medium with 1% BSA and seeded into 5 μm pore size transwell inserts (Corning) of a 24-well tissue culture plate containing 500 ml RPMI/BSA medium with CCL4 (R&D) and CCL5 (R&D) at a final concentration of 150 ng/mL. After incubation for 3 h, migrated $CD103^+$ cDC1 or $CD8\alpha^+$ cDC1 cells in the lower compartment were collected and quantified by flow cytometry. Migration was calculated as fold change between CCL4/5-treated group over the control group.

Bioinformatic analysis of cancer patient data

RNA-seq by expectation-maximization (RSEM)-normalized expression of skin cutaneous melanoma (SKCM, n = 458) or sarcoma (n = 265) patient cohorts from GDC Pan-Cancer (PANCAN) dataset of The Cancer Genome Atlas (TCGA) were used. Hierarchical clustering of expression data was plotted as heatmaps using the 'gplots' package (version 3.0.1), where red indicates higher, and blue indicates lower expressions relative to the mean expression per gene. For generation of gene expression signatures, normalized expression values were log2-transformed and ranked by the mean expression value of signature genes. The following gene signatures were used: STING expression (*Tmem173*), PolySTING-activated chemokines (CCL4, CCL5, CXCL9, CXCL10), cDC1 (CLEC9A, XCR1, CLNK, BATF3). Overall survival analyses were performed for the top and bottom quartile expression ranked values for selected genes or the ranked sum expression of gene signature and plotted for Kaplan-Meier curves using GraphPad Prism version 9.0.

Multiplex immunohistochemistry and multispectral analysis

Formalin-fixed and paraffin-embedded (FFPE) tumor tissues from patients post-NAC or -NAPC were collected for multiplex immunohistochemistry (mIHC) staining. mIHC staining was performed using a PerkinElmer Polaris 7-color Technology Kit according to the manufacturer's instructions. Spectra Systems was used to scan and photograph multispectral images at 20× magnification with the same exposure times with 10 randomly fields in each stained slides selected. The multispectral images were split and segmented using InForm image analysis software. Individual cells were identified with a nuclear segmentation algorithm using DAPI staining, together with a cellular mask around each nucleus to quantitate surface marker expression for each cell.

Statistical analysis

Statistical analysis was performed using Microsoft Excel and GraphPad Prism Version 9.0. Values reported in figures are expressed as the standard error of the mean, unless otherwise indicated. For normally distributed datasets, we used 2-tailed Student's t test between two comparison groups, one-way ANOVA among three or more groups, and two-way ANOVA followed by Bonferroni's multiple comparison test in tumor growth curves. For survival analysis, p values were calculated using the Log Rank test. p values > 0.05 were considered not significant (ns), p values < 0.05 were considered significant. * p value < 0.05, ** p value < 0.01, *** p value < 0.001, **** p value < 0.0001.

Supplementary Materials

Figs. S1 to S10

Table S1

Supplementary data file S1. Raw data excel file

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Acknowledgements We are grateful for suggestions and technical supports from Gao lab members, especially Z. T. Bennett, Q. Feng, J. Chen, W. Li and J. Wilhelm. This work was supported by grants from the National Institutes of Health (U54 CA244719, R01CA216839 to J.G.), National Natural Science Foundation of China (U20A20375 to J.W.), Cancer Prevention and Research Institute of Texas (RP220150 to J.G.) and Mendelson-Young Endowment in Cancer Therapeutics.

Author contributions J.W. and S.L. planned the study under the guidance of J.G., performed majority of the experiments, and analyzed the data. M.W. established the *XCR1*^{cre}*STING*$^{fl/fl}$ mice with the help of Z.S. and performed the DC-T cell priming experiments in the MC38-OVA tumor model. X.W. carried out PSC7A synthesis and PolySTING formulation. S.C. performed bioinformatic analyses and some RT-PCR measurements. X.R. contributed to the clinical sample collection and mIHC methodology. Y.X.F. and N.Y. assisted with multiple gene-knockout and immune-cell-dependent animal studies. S.L., J.W., M.W., G.H., B.D.S., Y.X.F. and J.G. prepared and revised the manuscript.

Competing interests B.D.S. and J.G. are co-founders, stockholders, and royalty recipients of OncoNano Medicine, Inc. G.H. is a scientific advisor and royalty recipient of OncoNano Medicine, Inc.

Data and materials availability: All materials used or generated in this study are available to researchers following appropriate standard material transfer agreements. All data are available in the main text or the supplementary materials.

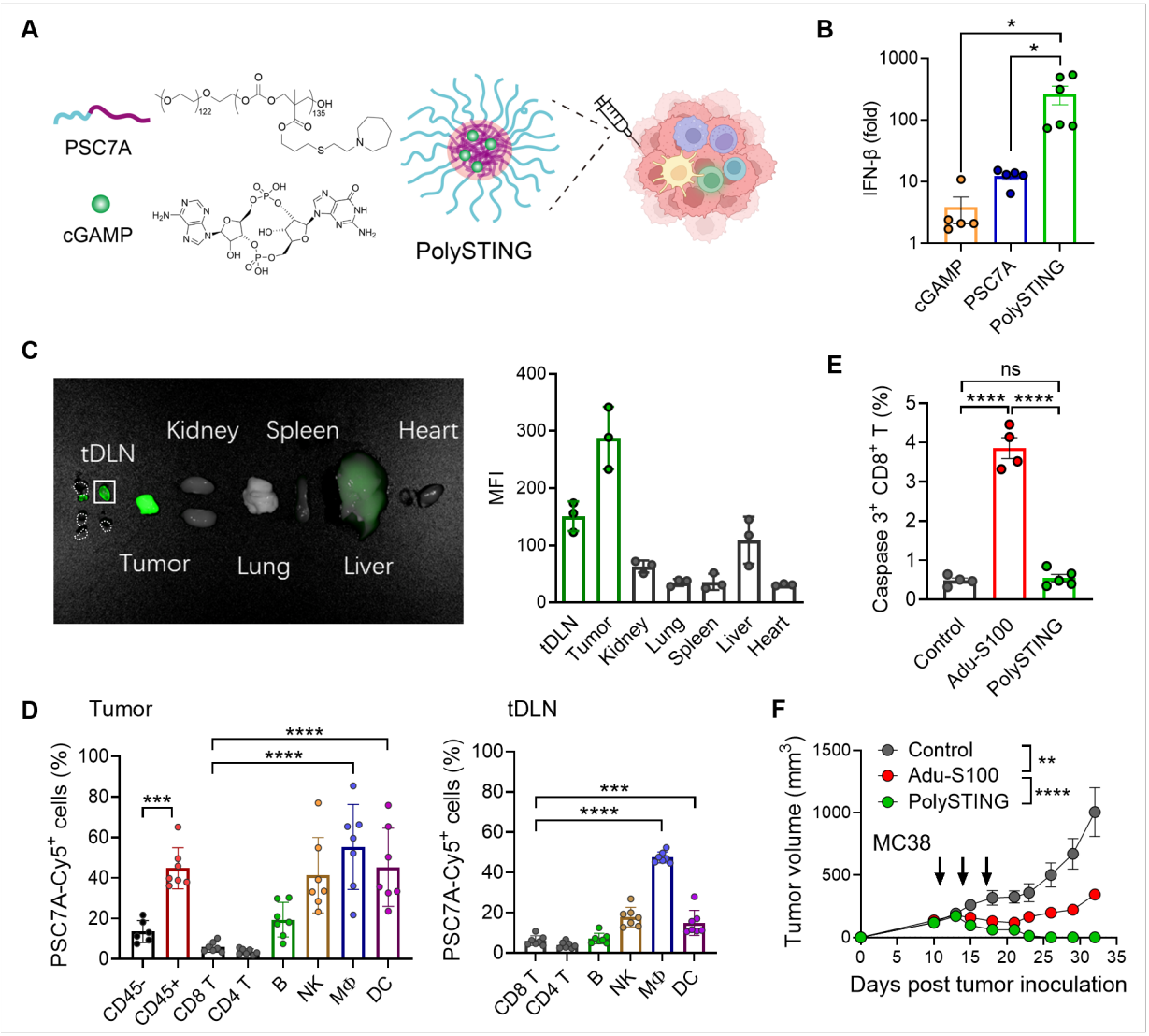

Fig. 1. Design and characterization of a polymeric STING-activating nanoparticle (PolySTING) for myeloid tropic STING activation. (**A**) Schematic overview of PolySTING micelle nanoparticles with dual STING activation by 2', 3'-cGAMP and PSC7A polymer. cGAMP is encapsulated in micelles self-assembled from pH-sensitive PSC7A polymers. (**B**) IFN-β-luciferase expression in ISG-THP1 cells after free cGAMP, PSC7A polymer, or PolySTING treatment over 24 hours. Equivalent cGAMP concentrations (5 μM) were used in these studies. n=5 independent experiments in cGAMP and PSC7A groups, and n=6 in PolySTING group. (**C**) *Ex vivo* imaging and tissue quantification of fluorescence intensity in MC38 tumors 24 hours post intratumoral administration of Cy5-labeled PolySTING. (**D**) Percentage of $Cy5^+$ cells amongst cell populations in the MC38 tumors and tumor-draining lymph nodes following intratumoral administration of Cy5-labeled PolySTING (n=7). (**E**) Percentage of caspase 3^+ $CD8^+$ T cells after intratumoral administration of PolySTING or Adu-S100 at the same dose (50 μg per mouse). n=4 in control and Adu-S100 groups, and n=5 in PolySTING group. (**F**) Antitumor efficacy of PolySTING or Adu-S100 in MC38 tumor bearing mice (n=6). All the data are represented as mean ± SEM. Statical significance was calculated by one-way ANOVA in B,D,E, or two-way ANOVA in F. ns: no significant difference, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

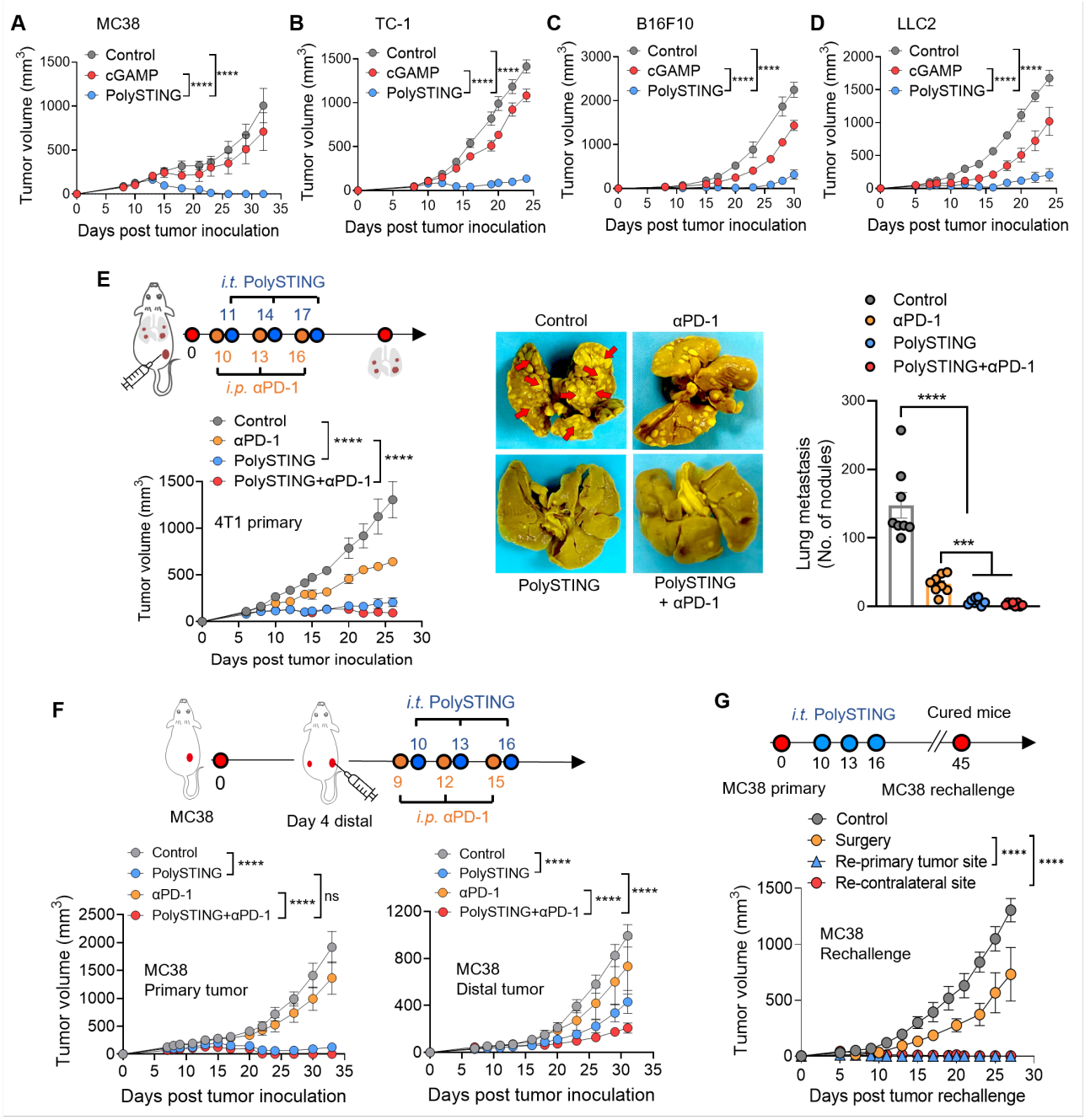

Fig. 2. Antitumor efficacy of PolySTING against established, metastatic, and distal tumors. (A-D) Growth inhibition curves of MC38 (n=5 or 7), TC-1 (n=8 or 13), B16F10 (n=5 or 11), or LLC2 tumors (n=6 or 10) following PolySTING treatment in C57BL/6 mice. (**E**) Primary tumor growth and metastasis to the lung in Balb/c mice bearing orthotopic triple negative 4T1 breast tumor after PolySTING and anti-PD-1 treatment (n=8). (**F**) Experimental schematic (top) and tumor growth curves (bottom) of injected primary and untreated contralateral tumors in C57BL/6 mice bearing MC38 tumors after PolySTING and anti-PD-1 treatment. (**G**) Experimental schematic (top) and tumor growth curves (bottom) of MC38 tumor-bearing mice cured by PolySTING (n=7) or surgical resection (n=5) rechallenged at day 45. All the data are represented as mean ± SEM. Statical significance was calculated by two-way ANOVA, ns: no significant difference, ***: $p<0.001$, ****: $p<0.0001$.

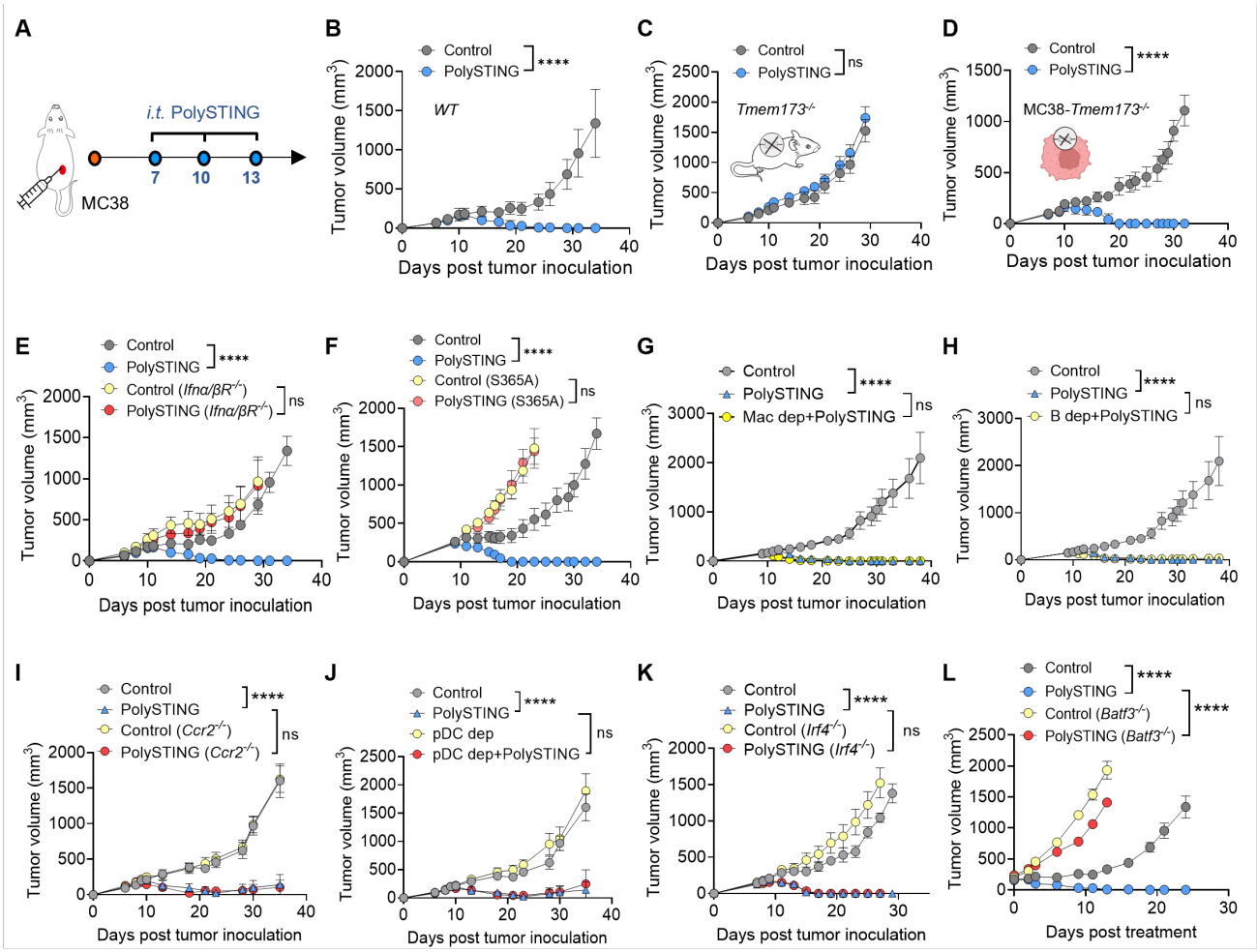

Fig. 3. Identification of cDC1 for STING-mediated tumor rejection. (**A**) Experiment schematic, (**B-D**) Tumor growth curves in wildtype (n=6 or 8), host STING knockout (*Tmem173*$^{-/-}$, n=7) or cancer STING knockout (MC38-*Tmem173*$^{-/-}$, n=5 or 7) C57BL/6 mice bearing MC38 tumors after PolySTING treatment or glucose control. Tumor growth of (**E**) Ifnα/β receptor knockout (*Ifnα/βR*$^{-/-}$, n=6) or (**F**) STING^{S365A} mutant mice (n=4) bearing MC38 tumors after PolySTING treatment. Tumor growth in wildtype mice with antibody blockade of (**G**) macrophages (anti-CSF1R, n = 5), (**H**) B cells (a mixture of anti-CD19/CD22/B220, n = 5), and transgenic mice with depletion of different DC subtypes, including (**I**) MoDC (*Ccr2*$^{-/-}$, n = 5), (**J**) plasmacytoid DC (pDC, anti-CD317, n = 5), (**K**) cDC2 (*Irf4*$^{-/-}$, n = 5), (**L**) cDC1 (*Batf3*$^{-/-}$, n = 7) mice, after PolySTING treatment. In all experiments, mice were intratumorally treated with PolySTING when the tumor volume reached around 200 mm^3 in MC38 tumors. Data are represented as mean ± SEM. Statical significance was calculated by two-way ANOVA, ns: no significant difference, ***: p<0.001, ****: p<0.0001.

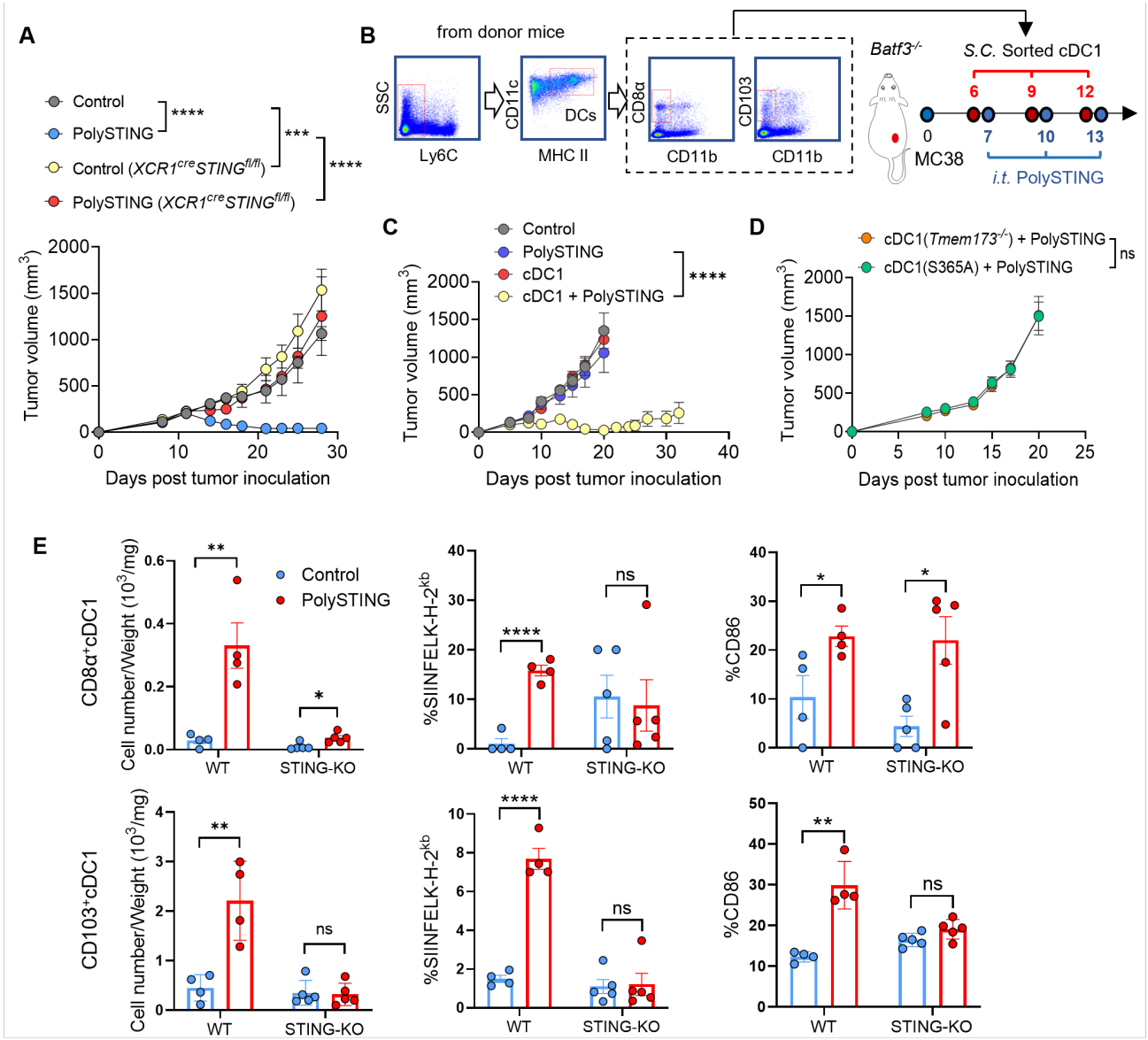

Fig. 4. Direct STING signaling in cDC1 is essential for PolySTING-mediated antitumor immunity. (**A**) Tumor growth in conditional knockout of STING in cDC1s (*XCR1*^{cre}*STING*$^{fl/fl}$) mice bearing MC38 tumors after PolySTING treatment (n=5). (**B**) Flow plots and experimental schematic, (**C-D**) MC38 tumor growth in *Batf3*$^{-/-}$ mice with or without PolySTING treatment after the mice were subcutaneously transferred with cDC1s from (C) STING wildtype mice (n=5), (D) STING knockout (n=5) or STING^{S365A} mutant mice (n=6). Mice were intratumorally treated with PolySTING when the tumor volume reached around 200 mm^3. cDC1s were sorted from BMDCs of donor mice. (**E**) Cell number per tumor weight, percentage of antigen (SIINFEKL) presentation and CD86 expression on CD8α$^+$ cDC1s or CD103$^+$ cDC1s with control or PolySTING treatment (n=4 or 5 mice per group). Tumor tissues were harvested and analyzed by flow cytometry at 24 hours after treatments. All the data are represented as mean ± SEM. Statical significance was calculated by two-way ANOVA in A-D, and two-tail t test in E. ns: no significant difference, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

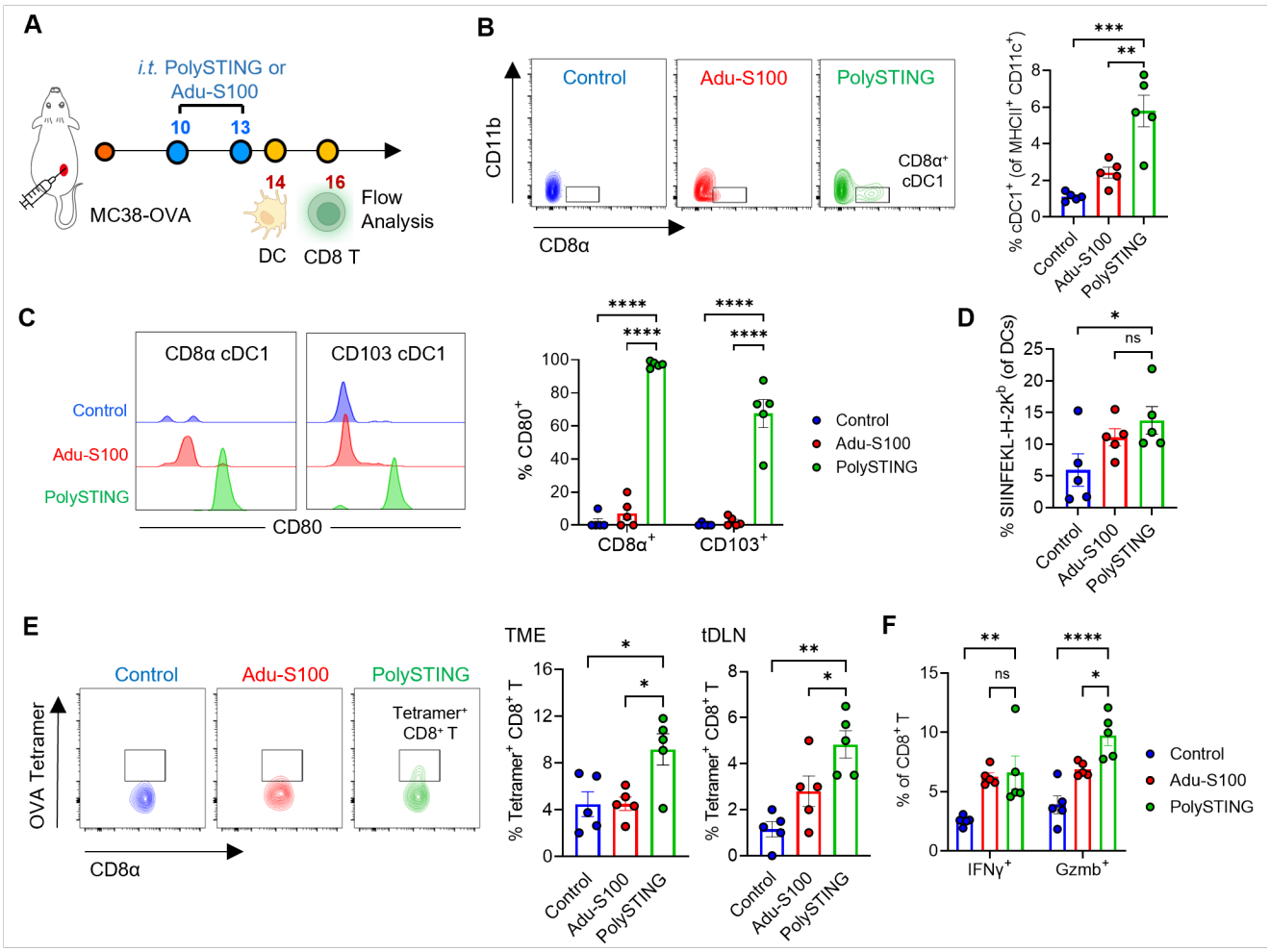

Fig. 5. STING signaling boosts cDC1 activation and priming of CD8⁺ T cells. (**A**) Schematic overview of experimental design using MC38-OVA tumor bearing mice. PolySTING or Adu-S100 were intratumorally administered on day 10 and 13 when average tumor volumes reached 150 mm^3. Dendritic cells and $CD8^+$ T cells were harvested on day 14 and 16, respectively, for flow cytometry analysis. (**B**) Representative flow plots of $CD8\alpha^+$ cDC1 (gated on $CD45^+CD11c^+MHCII^+CD11b^-$) and quantification of percentage of cDC1 in total DC population. (**C**) Representative flow cytometric histograms of the costimulatory marker CD80 (gated on $CD45^+CD11c^+MHCII^+CD11b^-CD8\alpha^+$ or $CD103^+$ cDC1) and quantification of CD80 percentage in $CD8\alpha^+$ and $CD103^+$ cDC1s. (**D**) Percentage of antigen (SIINFEKL) presentation on $H\text{-}2K^b$ complex of DCs in response to PolySTING or Adu-S100. (**E**) Representative flow cytometric plots of OVA-specific $CD8^+$ T cells from the tumor and tumor-draining lymph nodes in response to PolySTING or Adu-S100 treatment. (**F**) Percentage of interferon-γ (IFNγ) and granzyme b (Gzmb) expressing $CD8^+$ T cells in response to PolySTING or Adu-S100. All the data are represented as mean±SEM, n=5 mice in all experiments. Statical significance was calculated by two-way ANOVA, ns: no significant difference, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

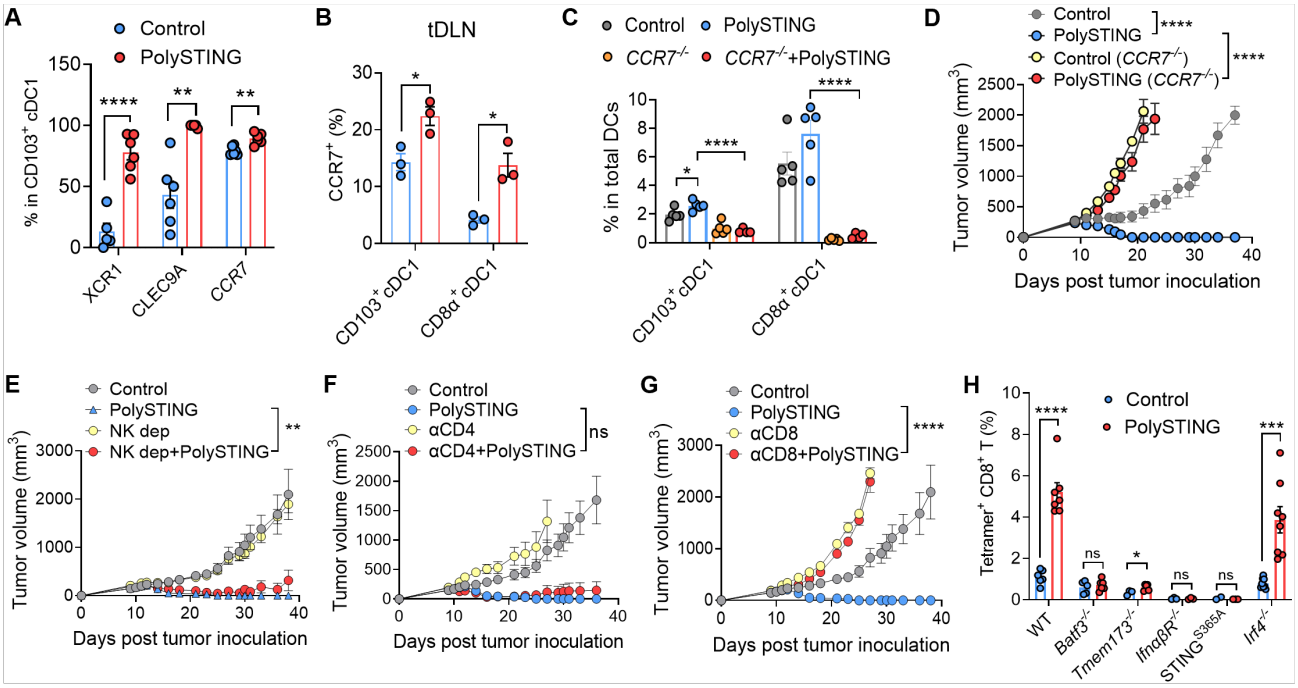

Fig. 6. PolySTING-mediated migration of cDC1 and effect of T cells on antitumor efficacy. (**A**) Percentage of XCR1, CLEC9A, and CCR7 expression on cDC1s in MC38 tumors 18 h after intratumoral PolySTING treatment (n =6). (**B**) Expression of CCR7 on cDC1s in tumor-draining lymph nodes (tDLN) after PolySTING treatment (n=3). (**C**) cDC1 percentage in total DCs in MC38 tumors and (**D**) tumor growth after PolySTING treatment in wildtype or CCR7 knockout (*CCR7*$^{-/-}$) mice (n=5). MC38 tumor growth in C57BL/6 mice after depletion of (**E**) NK cells (anti-NK1.1), (**F**) CD4^{+} T cells (anti-CD4), (**G**) CD8^{+} T cells (anti-CD8a), n=5 mice. (**H**) Tetramer^{+} CD8^{+} T cells from tDLN of wildtype and different genetic knockout mice with or without PolySTING treatment. n=6 WT, 5 *Batf3*$^{-/-}$, 5 *Tmem173*$^{-/-}$, 3 *IfnαβR*$^{-/-}$, 3 *STING*^{S365A}, 8 *Irf4*$^{-/-}$ mice. Mice receiving 5% glucose were used as controls. All the data are represented as mean±SEM. Statical significance was calculated by two-tail t test in A, B, H, one-way ANOVA in C, or two-way ANOVA in D-G. ns: no significant difference, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

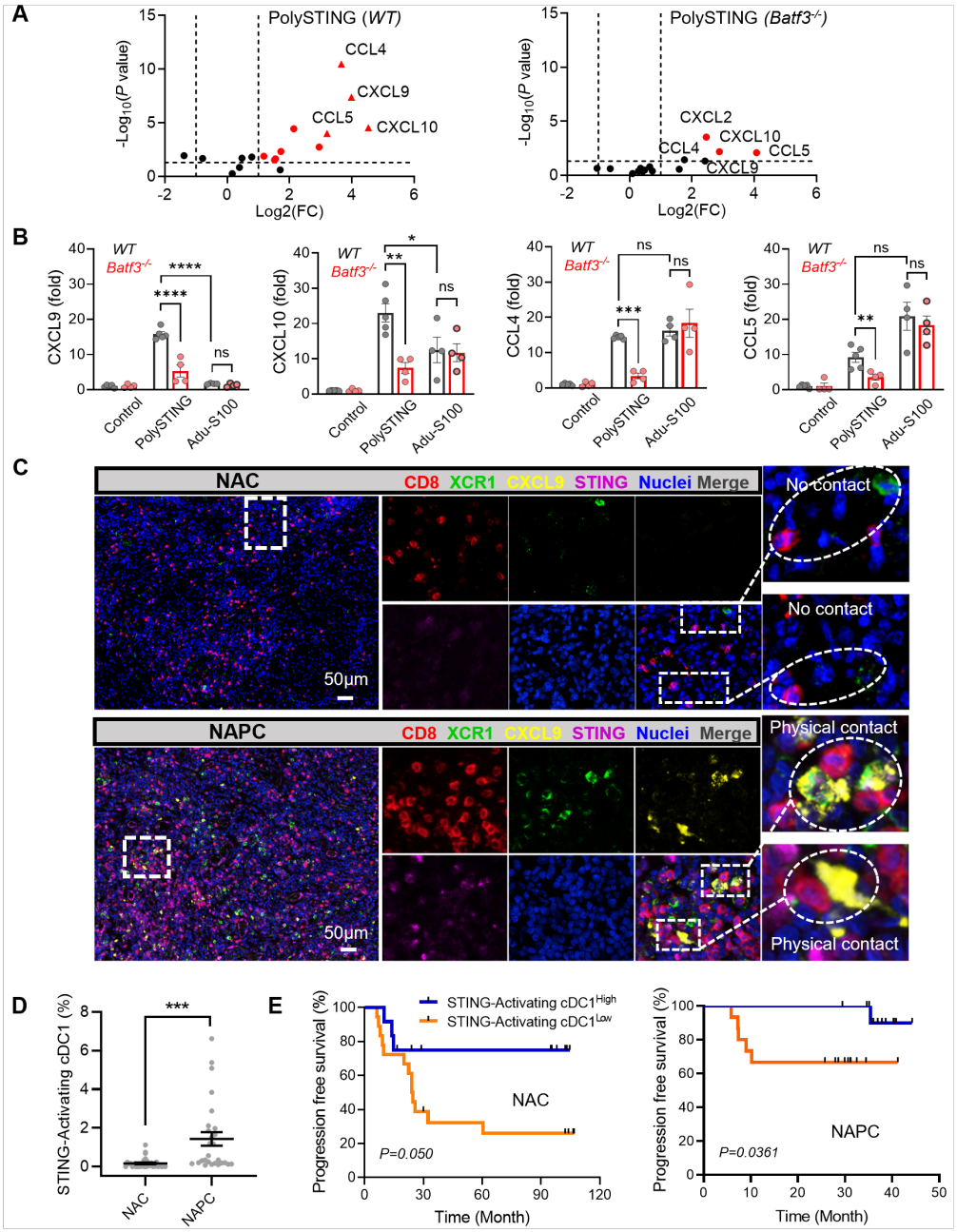

Fig. 7. STING-activating cDC1 signature is associated with cancer patient survival. (**A**) Quantification of chemokine profile of MC38 tumors from wildtype or *Batf3*$^{-/-}$ mice after PolySTING treatment. (**B**) Comparison of expression levels of chemokines CCL4/5 and CXCL9/10 in wildtype versus *Batf3*$^{-/-}$ mice after indicated treatments. Data are represented as mean ± SEM, n=5 for wildtype and n=4 for *Batf3*$^{-/-}$ mice. (**C**) Representative images of mIHC staining of NSCLC tumors from either NAC or NAPC-treated patients. (**D**) Percentage of STING activating cDC1 was quantified and compared between NAC and NAPC-treated patients. (**E**) STING-activating cDC1 signature shows positive correlation with progression free survival in both NSCLC patients treated with either NAC or NAPC. Statical significance was calculated by one-way ANOVA in B, two-tail t-test in D, and Logrank test in E. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.