STING activation of tumor endothelial cells initiates spontaneous and therapeutic antitumor immunity

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Spontaneous CD8 T-cell responses occur in growing tumors but are usually poorly effective. Understanding the molecular and cellular mechanisms that drive these responses is of major interest as they could be exploited to generate a more efficacious antitumor immunity. As such, stimulator of IFN genes (STING), an adaptor molecule involved in cytosolic DNA sensing, is required for the induction of antitumor CD8 T responses in mouse models of cancer. Here, we find that enforced activation of STING by intratumoral injection of cyclic dinucleotide GMP-AMP (cGAMP), potently enhanced antitumor CD8 T responses leading to growth control of injected and contralateral tumors in mouse models of melanoma and colon cancer. The ability of cGAMP to trigger antitumor immunity was further enhanced by the blockade of both PD1 and CTLA4. The STING-dependent antitumor immunity, either induced spontaneously in growing tumors or induced by intratumoral cGAMP injection was dependent on type I IFNs produced in the tumor microenvironment. In response to cGAMP injection, both in the mouse melanoma model and an ex vivo model of cultured human melanoma explants, the principal source of type I IFN was not dendritic cells, but instead endothelial cells. Similarly, endothelial cells but not dendritic cells were found to be the principal source of spontaneously induced type I IFNs in growing tumors. These data identify an unexpected role of the tumor vasculature in the initiation of CD8 T-cell antitumor immunity and demonstrate that tumor endothelial cells can be targeted for immunotherapy of melanoma.

STING | tumor endothelial cells | type I IFNs | CD8 T cells | antitumor immunity

Metastatic melanoma is a highly aggressive cancer with a fast increasing incidence worldwide. Unless diagnosed early and surgically resected, the disease becomes metastatic and life threatening. Both chemotherapy and irradiation are ineffective. Novel therapies that target oncogenic drivers have brought some improvements, but tumor cells escape regularly (1). Melanoma is a prototypical immunogenic tumor, as shown by the occurrence of spontaneous CD8 T-cell responses that drive tumor regressions and by the identification of CD8 T cells that recognize melanoma antigens (2, 3). Although many immunotherapeutic strategies have been developed to induce such responses, clinical efficacies have been poor. More recently, "checkpoint blockade" therapies that target T-cell–inhibitory pathways mediated by CTLA4 (4) and PD1 (5) have yielded encouraging clinical results and demonstrated that spontaneous CD8 T-cell responses in tumors can be boosted to treat melanoma.

The mechanisms that drive spontaneous antitumor immune responses are poorly understood. Type I IFNs (IFN-α and IFN-β) may play a role as the expression type I IFN-related genes in primary melanoma has been associated with spontaneous tumor regressions (6) and correlated to the tumor infiltration by specific CD8+ T cells (6, 7). Furthermore, the lack of type I IFN signaling or IFN-β expression inhibited the generation of tumor-specific CD8 T cells and accelerated tumor growth in a murine melanoma model (7–9).

Type I IFNs are typically induced upon recognition of nucleic acids in intracellular compartments. Several cytosolic DNA receptors have been identified and include DNA-dependent activator of IFN-regulatory factors (DAI) (10), gamma-interferon-inducible protein-16 (IFI16) (11), and the helicase DEAD (Asp-Glu-Ala-Asp) box protein 41 (DDX41) (12). These cytosolic receptors trigger IFN-β production via a signaling cascade that involves the master adaptor molecule stimulator of IFN genes (STING), which binds to tank-binding kinase 1 (TBK1) and induces phosphorylation of the interferon regulatory factor 3 (IRF3) (13). STING associates weakly to dsDNA (14) but strongly binds the endogenous cyclic dinucleotide GMP-AMP (cGAMP) synthesized by the cGMP-AMP synthase (cGAS) (15, 16).

Recently, spontaneous STING activation was found to be required for the spontaneous induction of antitumor immunity (17). Activation of STING occurred via tumor DNA-dependent cGAS activation and generation of endogenous cGAMP (17, 18). However, the cellular mechanism underlying this response and whether this mechanism could be exploited to generate more efficient antitumor immune responses is currently unknown.

Here, we show that intratumoral injection of exogenous cGAMP enhanced STING activation and strongly promoted the generation of antitumor CD8 T-cell responses leading to efficient growth control of injected and contralateral tumors. This response was dependent on IFN-β produced by tumor endothelial cells. In fact,

Significance

Tumor recognition by the immune system can occur spontaneously but has usually little impact on tumor growth. However, the cellular and molecular mechanisms that drive these responses could be exploited therapeutically to generate efficacious antitumor immunity. Here, we show that stimulator of IFN genes (STING), a molecule involved in cytosolic DNA sensing and required for the generation of spontaneous antitumor immune responses, can be targeted by intratumoral injection of cGAMP to boost antitumor immunity and to control tumor growth. The immune response induced by therapeutic but also spontaneous STING activation was dependent on type I IFN produced by endothelial cells in the tumor microenvironment, unraveling an unexpected role of the tumor vasculature in the initiation of spontaneous and therapeutic antitumor immunity via STING.

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endothelial cells were found to be the principal producers of type I IFN in response to both spontaneous and enforced STING activation, suggesting a role of the tumor vasculature in the initiation of antitumor immunity.

Results

Intratumoral Injection of cGAMP Promotes the Induction of CD8 T-Cell Responses and Efficiently Delays Growth of Injected Tumors. Initially, we established a mouse melanoma model to study STING-dependent induction of antitumor immunity. We engrafted B16F10 melanoma cells in wild-type mice or STING-deficient mice ($STING^{gt/gt}$) and measured tumor-infiltrating CD8 T cells as well as tumor size. CD8 T cells spontaneously infiltrating tumors were drastically reduced and tumor growth was accelerated in $STING^{gt/gt}$ compared with WT mice (Fig. 1 *A* and *B*). Because this spontaneous STING-dependent CD8 T-cell response was insufficient to control the growth of tumors, we sought to investigate whether the intratumoral injection of the STING agonist cGAMP could be used therapeutically to enhance this response. We injected cGAMP into B16F10 melanoma at day 5 and day 10 after engraftment and measured CD8 T-cell in the tumors along with tumor size. Compared with control injected tumors, tumors injected with cGAMP showed a mean of fourfold increase of CD8 T cells in the tumor microenvironment (Fig. 1*A*) along with a significant delay in tumor growth (Fig. 1*B*). These effects were completely abolished in $STING^{gt/gt}$ mice (Fig. 1 *A* and *B*). cGAMP also increased tumor-infiltrating CD8 T-cell numbers and delayed tumor growth when injected into engrafted melanoma derived from Tyr::*N*-$ras^{(Q61K)}$$INK4A^{-/-}$ mice (19) (Fig. S1) and B-$raf^{(V600E/+)}$ $PTEN^{-/-}$ $CDKN2A^{-/-}$ mice (20) (Fig. S1). Tumor-infiltrating CD8 T cells displayed an effector phenotype (CD44hi CD62Llow) and were able to produce IFNγ. Compared with control-injected tumors, higher numbers of perforin-expressing CD8 T cells were found in cGAMP-injected tumors (Fig. S2) suggesting the induction of cytotoxic effector CD8 T cells. These tumor-infiltrating CD8 T cells appeared to be tumor antigen-specific as OVA specific CD8 T cells were detected in the tumors when OVA was used as a model antigen expressed by tumors (Fig. S2). Thus, intratumoral cGAMP promotes the generation of Ag-specific cytotoxic CD8 T cells that infiltrate tumors. Interestingly, intratumoral cGAMP injection also induced high numbers of infiltrating CD4 T cells in B16F10, Tyr::*N*-$ras^{(Q61K)}$$INK4A^{-/-}$, and B-$raf^{(V600E/+)}$ $PTEN^{-/-}$ $CDKN2A^{-/-}$ melanoma (Fig. S3). Intratumoral cGAMP improved survival of tumor-bearing mice as shown by the fact that 31% of WT mice treated with intratumoral cGAMP survived up to 3 mo, whereas control mice all died within 3 wk (Fig. 1*C*). The antitumor efficacy of intratumoral cGAMP was further enhanced by concomitant blockade of CTLA4 and PD1. In fact, the anti-CTLA4+anti-PD1 combo increased the antitumor activity of cGAMP in a dose-dependent manner (Fig. S4) and led to a complete inhibition of tumor growth for up to 23 d post-implantation (Fig. 1*D*). These data indicate that enforced STING activation by intratumoral cGAMP injection can potently increase the generation of Ag-specific effector CD8 T cells infiltrating the tumor and efficiently control its growth.

Intratumoral STING Activation Leads to Systemic CD8 T-Cell–Mediated Antitumor Immunity That Controls the Growth of Distant Tumors. We next investigated whether, via the induction of CD8 T-cell responses, intratumoral injections of cGAMP could induce systemic antitumor immunity. First, mice bearing skin tumors that had been injected with cGAMP, received i.v. B16F10 tumor cells to induce lung metastases. Ten days later, mice were killed and the number of melanoma metastases was counted in the lungs. Intratumoral injection of cGAMP potently reduced the number of lung metastases (Fig. 2*A*), suggesting the induction of systemic immunity that inhibits metastasis formation. To confirm this, we engrafted B16F10 tumors into opposite flanks of the same mouse followed by treatment of only one tumor while leaving the contralateral one untreated. We found that intratumoral cGAMP treatment not only delayed the growth of injected tumors, but also delayed the growth of contralateral tumors (Fig. 2*B*). This effect was not limited to B16 melanoma, as intratumoral injection of cGAMP also delayed the growth of injected and contralateral MC38 colon tumor cells implanted into the skin (Fig. S5). Antibody-mediated CD8 T-cell depletion completely abolished the effect of cGAMP on contralateral tumor (Fig. 2*B*) but only had a partial effect on cGAMP-injected tumors (Fig. 2*C*). These data suggest that intratumoral cGAMP induces CD8 T-cell priming that drive systemic antitumor immunity controlling local and distant tumor growth. Interestingly, the local antitumor response to cGAMP is only partially dependent on these CD8 T cells, suggesting that additional mechanisms account for a direct antitumor activity.

The Antitumor Activity Induced by STING Is Dependent on Type I IFN Signaling. Because STING has been associated with type I IFN induction (21) we next sought to investigate the role of type I IFNs in mediating the antitumor CD8 T-cell response induced by cGAMP. As previously described (17), low levels of type I IFNs were spontaneously induced by STING signaling in growing tumors of WT mice as we detected the expression of the type I IFN-inducible genes *Mx2*, *Irf7*, and *Isg15* that were abolished in $STING^{gt/gt}$ mice (Fig. S6). Lack of type I IFN signaling in $IFNAR^{-/-}$ mice not only abolished the type I IFN signature in

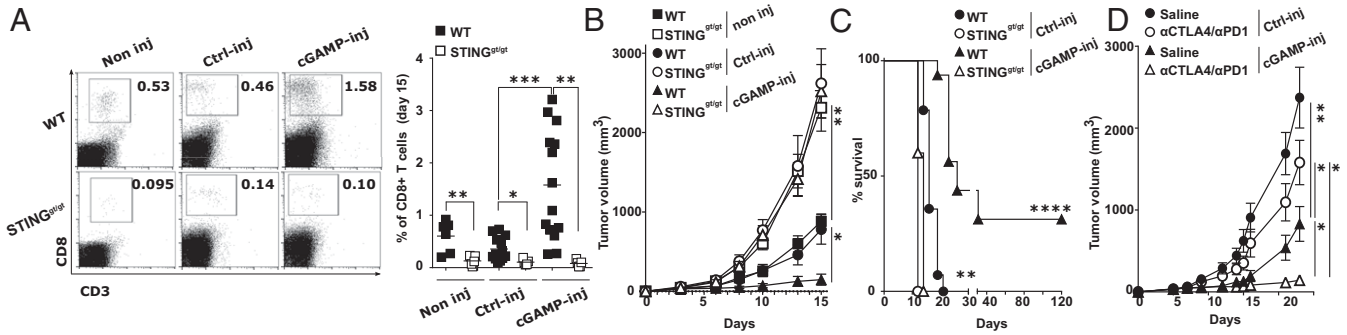

Fig. 1. Intratumoral cGAMP promotes CD8 T-cell responses and efficiently delays growth of injected tumors. (*A*–*C*) C57BL6 or $STING^{gt/gt}$ mice bearing established s.c. B16F10 tumors were either left untreated (non inj), injected intratumorally with cGAMP (cGAMP-inj), or injected with Lipofectamine alone (Ctrl-inj) at day 5 and day 10 postengraftment. Depicted are: (*A*) CD3+CD8+ T cells infiltrating tumors at day 15 postengraftment, measured by flow cytometry of tumor single cell suspensions. Representative plots are given (*Left*). Each symbol represents a separate mouse (*Right*). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by unpaired *t* test. (*B*) Tumor growth over time. Data are shown as mean tumor volume ± SEM of 5–6 mice per group, $*P < 0.05$, $**P < 0.01$ by two-way ANOVA. (*C*) Mouse survival monitored over time. Analysis include 5–16 mice per group, $**P < 0.01$, $****P < 0.0001$ by log rank (Mantel Cox) test. (*D*) Mice bearing s.c. B16 tumors were treated as described above in the setting of anti-CTLA4 and anti-PD1 antibody treatment. Graph depicts tumor growth over time. Data are shown as mean tumor volume ± SEM of seven mice per group. $*P < 0.05$, $**P < 0.01$ by two-way ANOVA.

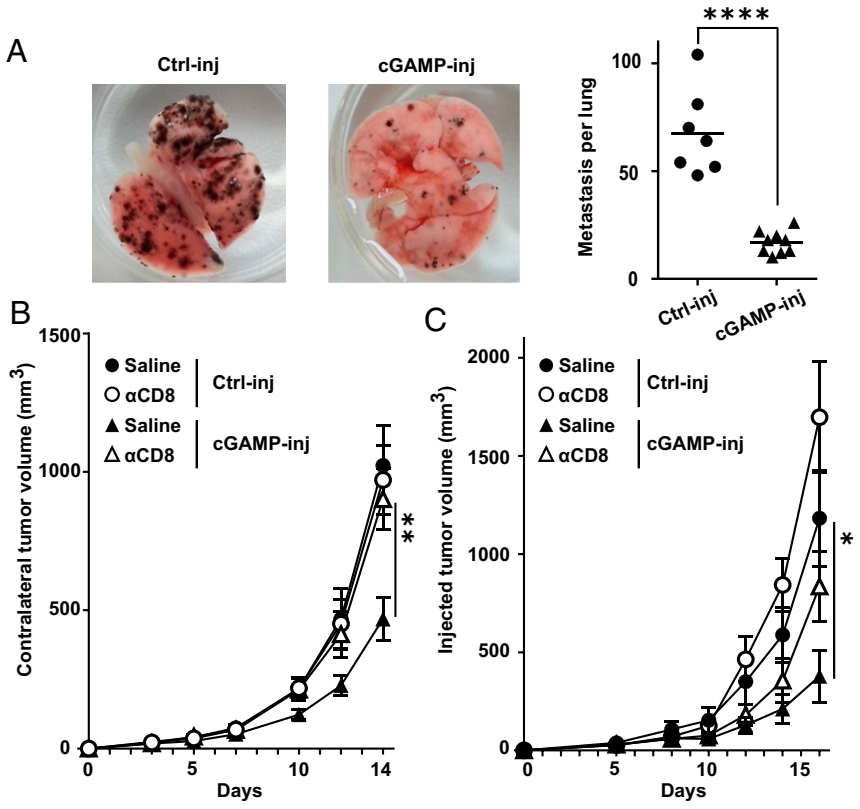

Fig. 2. Intratumoral STING activation leads to systemic CD8 T-cell–mediated antitumor immunity that controls the growth of distant tumors. (*A*) Mice bearing a 5-d established s.c. B16F10 tumor were treated by i.t. injection of cGAMP (cGAMP-inj) or Lipofectamine alone (Ctrl-inj) followed by i.v. injection of B16F10 melanoma cells. After 10 d, mice were killed and lung metastases counted macroscopically. Photographs of representative lungs are depicted (*Left*). Each symbol represents an independent mouse (*Right*); data are combined from two experiments. $^{****}P < 0.0001$ by unpaired *t* test. (*B* and *C*) Mice bearing two s.c. B16F10 tumors in opposite flanks were treated by i.t. injection of cGAMP (cGAMP-inj) or Lipofectamine alone (Ctrl-inj) in only one tumor. CD8-depleting antibody or saline were injected i.p. Graph depicts tumor growth over time of contralateral not injected tumors (*B*) and injected tumors (*C*). Data represent the mean tumor volume ± SEM of 9–10 mice per group pooled from two experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ by two-way ANOVA.

tumors, but also abrogated CD8 T-cell responses in the tumors (Fig. S6). We then sought to investigate whether the same type I IFN-dependent mechanism would underlie the strong antitumor CD8 T-cell responses observed following cGAMP injection. First, we measured type I IFN activity induced by intratumoral cGAMP. Strong type I IFN activity was detected starting 1 h after injection, peaking between 2 h and 4 h, and declining thereafter (Fig. 3*A*). mRNA expression analysis 4 h after injection revealed expression of *Ifn-β1* but not of *Ifn-α* genes such as *Ifn-α2*, *Ifn-α5*, and *Ifn-α6* (Fig. 3*B*), indicating that intratumoral STING activation triggers an early type I IFN activity via the induction of *Ifn-β1* expression. We then assessed the role of IFN-β in the local and systemic antitumor activity induced by cGAMP. Treatment of WT mice with blocking anti-IFNAR antibodies or the use of $IFNAR^{-/-}$ mice to block IFNAR signaling completely abolished intratumoral CD8 T-cell numbers and antitumor activities in both injected and contralateral tumors (Fig. 3 *C* and *D*). Interestingly, despite being only partially CD8 T-cell dependent, the local antitumor activity induced by cGAMP injection was entirely mediated by type I IFNs. To test whether IFN-β expression had a direct inhibitory activity on the growth of B16 tumor cells, we generated extracts from cGAMP-injected tumors and tested their ability to block the growth of B16 tumor cells with or without neutralizing antibodies to IFNAR. These tumor extracts were indeed able to block the growth of B16 cells in a type I IFN-dependent manner (Fig. S7), a finding that is consistent with previous reports showing that type I IFNs block proliferation and induce apoptosis of B16 cells (22, 23). Together these data indicate that IFN-β is required for the generation of tumor-infiltrating CD8 T cells and for the antitumor activity induced by intratumoral cGAMP.

Endothelial Cells Are the Principal IFN-β Producers in Response to Therapeutic STING Activation in Tumors. To identify the cell type responsible for the STING-mediated induction of IFN-β production in the tumor microenvironment, we performed intracellular IFN-β staining of tumor-cell–derived single cell suspensions generated after intratumoral cGAMP injection. Flow cytometry analysis revealed a small population of IFN-β–producing cells in the tumor microenvironment of engrafted wild-type mice that was not present in tumors engrafted in STING-deficient mice (Fig. 4*A*). Phenotypical analysis of IFN-β–producing cells revealed that IFN-β was produced by a homogeneous population that did not express lineage markers for T cells, B cells, NK cells, DC, monocyte/macrophages nor neutrophils (CD3, CD19, NKp46, CD11C, CD11b, and Ly6G, respectively) (Fig. 4*B*). IFN-β–producing cells expressed low levels of the hematopoietic marker CD45 and, surprisingly, expressed the specific endothelial cell markers CD31, VE-Cadherin, and VEGFR-2 (Fig. 4*B*). To confirm that endothelial cells are the principal target of STING activation in the tumor microenvironment, we performed immunofluorescent staining for IFN-β on cGAMP-injected tumors engrafted in wild-type mice or STING-deficient mice. IFN-β was always found to be associated with the VEGFR2+ and CD31+ tumor endothelial cells (Fig. 4*C* and Fig. S8) and not produced by CD45+ infiltrating immune cells including CD11c+ dendritic cells (DCs) (Fig. 4*C*). Importantly, IFN-β was not detected in cGAMP-injected tumors engrafted in $STING^{gt/gt}$ mice (Fig. 4*C* and Fig. S8). Our data suggest that endothelial cells and not DCs are the principal type IFN-producing cell type in the tumor in response to STING activation. These data were confirmed by the finding that the IFN-β induction in response to intratumoral cGAMP was unaltered in CD11c-depleted mice (Fig. S9). More specifically, we confirmed that plasmacytoid dendritic cells (pDCs), which are the principal type I IFN-producing cells during antiviral immune responses, were not involved in the antitumoral activity induced by cGAMP as inhibition of tumor growth was retained upon pDC depletion in hBDCA2-DTR mice (Fig. S9). Thus, our data identify tumor endothelial cells as the principal type I IFN producers in response to therapeutic STING activation by cGAMP.

To determine whether cGAMP could also activate STING in human melanoma, we injected cGAMP into freshly resected melanoma skin metastases. Intratumoral cGAMP induced high levels of IFN-β expression in skin melanoma metastases compared with control injected melanoma (Fig. 4*D*). Immunofluorescence

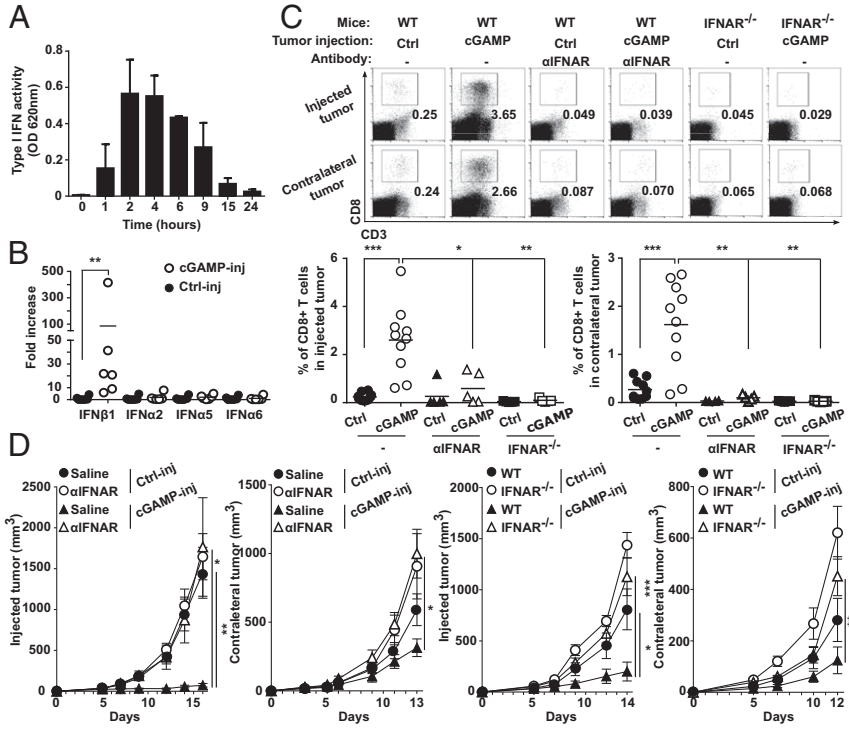

Fig. 3. Intratumoral cGAMP induces expression of IFN-β that drives local and systemic antitumor immunity. (*A* and *B*) Mice bearing 5-d established s.c. B16F10 tumors were treated by i.t. injection of cGAMP (cGAMP-inj) or Lipofectamine alone (Ctrl-inj). Depicted are: (*A*) Type I IFN activity in tumor extracts at the indicated time after treatment. (*B*) *Ifn-β1*, *Ifn-α2*, *Ifn-α5*, and *Ifn-α6* mRNA expression in tumors 4 h after treatment. Each symbol represents an independent mouse, $^{**}P < 0.001$ by unpaired *t* test. (*C* and *D*) B16F10 cells were implanted s.c. into opposite flanks of WT or IFNAR$^{-/-}$ mice followed by i.t. injection of cGAMP (cGAMP-inj) or Lipofectamine alone (Ctrl-inj) in only one tumor. WT mice were also treated with a neutralizing αIFNAR antibody. Depicted are: (*C*) CD3+CD8+ T cells infiltrating tumors at day 15 postengraftment, measured by flow cytometry of tumor single cell suspensions. Representative plots are given (*Upper*); each symbol represents an independent mouse (*Lower*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by unpaired *t* test. (*D*) Tumor growth over time of injected and contralateral tumors. Data are given as the mean tumor volume ± SEM with $n = 5$, representative of two independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-way ANOVA.

stainings confirmed that IFN-β was expressed predominantly by CD31+ endothelial cells in the tumor microenvironment. Thus, intratumoral cGAMP can induce type I IFNs by the human tumor vasculature (Fig. 4*E*).

Endothelial Cells Produce High Level of Type I IFNs upon STING Activation. To evaluate the capacity of endothelial cells to produce type I IFNs, we performed in vitro stimulation of murine endothelial cells with cGAMP or CpGs in comparison with total CD11c+ DCs, which have previously been suggested to be the main IFN producers in the tumor microenvironment (17). We found stronger type I IFN activity in endothelial cells stimulated by cGAMP compared with DCs (Fig. 5*A*). By contrast, type I IFN activity was higher in DCs stimulated with CpG compared with endothelial cells (Fig. 5*A*). Because cytosolic transfer of extracellular tumor-derived DNA can trigger activation of STING (17), we tested whether it could also trigger type I IFN production in endothelial cells and DCs. Lipofected tumor DNA was indeed a potent inducer of type I IFN activity via STING activation of endothelial cells but not DCs (Fig. 5 *A* and *B*). IFN-β and IFN-α mRNA expression analysis confirmed that STING activation induced a predominant IFN-β expression in endothelial cells compared with DCs, whereas IFN-α mRNA was hardly detectable in both cell types (Fig. 5*C*). Although poorly responding to STING activation by cGAMP, purified pDCs expressed high levels of IFN-α and IFN-β mRNA upon stimulation with CpG (Fig. 5*C*), consistent with their specialized function as type I IFN producers in response to endosomal TLR activation. Therefore, in vitro, endothelial cells appear to produce higher amounts of IFN-β in response to STING activation compared with DCs.

Endothelial Cells Are the Principal IFN-β Producers in Response to Spontaneous STING Activation in Tumors. Next we sought to determine whether endothelial cells also represent the main source of spontaneous type I IFN expression in growing tumors. mRNA expression analysis revealed that *Ifn-β1* was spontaneously induced in the tumors as early as day 3 after tumor engraftment, reaching maximal expression levels at day 5 (Fig. 6*A*). This time point was also characterized by the presence of a large number of CD45- CD31+ VEGFR2+ endothelial cells in the tumor, whereas CD45+ immune cells including CD8 T cells and CD11c+ DCs appeared later (Fig. 6 *B* and *C*). Immunofluorescence staining identified a rare population of IFN-β–expressing cells (Fig. S8), which always costained with endothelial cell markers and was completely absent in tumors of STING-deficient mice (Fig. 6*D*). These data suggest that tumor endothelial cells are also a main source of spontaneous type I IFN expression in the tumor microenvironment. Endothelial cell-derived type I IFNs preceded the infiltration of CD11c+ DCs and CD8 T cells in the tumor microenvironment, suggesting their role in initiating the antitumor response via type I IFN production.

Discussion

Our study demonstrates that intratumoral injection of cGAMP induces potent STING activation in the tumor microenvironment and thereby inhibits tumor growth by promoting natural CD8 T-cell responses against the tumor. The cGAMP-induced antitumor activity was found to be mediated by the STING-driven induction of IFN-β in the tumor microenvironment. Interestingly, tumor endothelial cells were the main producer of IFN-β in response to cGAMP injection in both mouse and human. Endothelial cells were also found to be the main source of spontaneous IFN-β production in growing tumors. Together these data indicate that the tumor vasculature plays a key role in the initiation of spontaneous and therapeutic CD8 T-cell immunity against the tumor via STING-dependent induction of type I IFNs.

Type I IFNs play a multifaceted role in the induction of antitumor immunity. First, type I IFNs enhance tumor-specific CD8 T cells priming by promoting the recruitment and activation of DCs at the tumor site (7, 8). Accordingly, type I IFNs were found to increase the number of DCs in the microenvironment of cGAMP-injected tumors and to stimulate DC maturation as we detected a higher levels of CD80 and CD86 in DCs in lymph nodes draining cGAMP-injected tumors (Fig. S10). Second, type I IFNs may increase CD8 T-cell infiltration into cGAMP-injected tumors (6) and directly promote their survival and proliferation in the tumor microenvironment (24). However, our data suggest that the potent local antitumor efficacy is not explained by increased CD8 T-cell infiltration of cGAMP-injected tumor but rather by a direct inhibitory effect of type I IFNs on the tumor itself (22, 23).

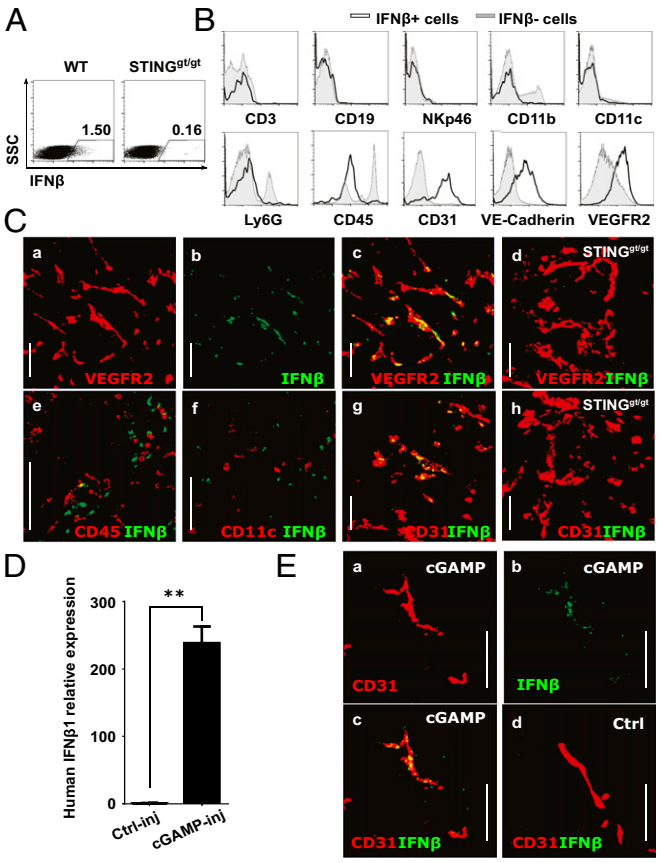

Fig. 4. Endothelial cells represent the main IFN-β producers upon intratumoral cGAMP injection in mouse and human. (*A*–*C*) WT or STING$^{gt/gt}$ mice bearing 5-d established s.c. B16F10 tumors were treated by i.t. injection of cGAMP plus Brefeldin A. Tumors were harvested after 5 h for flow cytometry and confocal microscopy analysis. (*A*) Intracellular IFN-β detection in tumor single cell suspensions. (*B*) Expression of CD3, CD19, NKp46, CD11b, CD11c, Ly6G, CD45, CD31, VE-cadherin, and VEGFR2 in IFN-β–producing cells (black line, gated as shown in *A* compared with nonIFN-β–producing cells (gray line). Data are representative of at least three independent experiments. (*C*) IFN-β expression on CD31 or VEGFR2 cells in tumor cryosections derived from WT (*a*–*c* and *e*–*g*) or STING$^{gt/gt}$ mice (*d* and *h*). (Scale bar, 100 μm.) (*D* and *E*) Resected human melanoma skin metastases were injected with 2′3′-cGAMP (cGAMP-inj) or Lipofectamine alone (Ctrl-inj) and cultured in the presence of Brefeldin A (only in *E*) for 5 h before analysis. Depicted are: (*D*) Quantitative real-time PCR analysis of *IFN-β1* mRNA expression in treated tumor explants. $^{**}P < 0.01$ by unpaired *t* test. (*E*) Confocal microscopy of IFN-β and CD31 stained on cryosections derived from treated tumor explants. (Scale bar, 100 μm.)

DCs are typically believed to be the main source of type I IFN during immune response. Unexpectedly, our study identifies endothelial cells and not DCs as the principal IFN-producing cell in response to both spontaneous and enforced STING activation in the tumor microenvironment. One reason for this is that endothelial cells possess an enhanced capacity to produce type I IFNs in response to STING activation compared with DCs. Confirming our data, previous studies found that in vitro-stimulated endothelial cells express more type I IFNs than other immune cells such as macrophages following STING activation by viruses (25). Another reason for the preferential type I IFN production by endothelial cells may be related to their relative abundance in the tumor compared with tumor-infiltrating immune cells. Indeed, at the time of intratumoral cGAMP injection, endothelial cells in the tumor microenvironment are fourfold more abundant than DCs. Interestingly, we found exclusive expression of IFN-β but did not detect any IFN-α in the tumor microenvironment. This may be related to the weak capacity of endothelial cells to produce IFN-α upon STING activation, but may also be related to the fact that IFN-α expression requires prior IFN-β–mediated up-regulation of IRF7 (26).

In spontaneously growing tumors IFN-β is also induced early at the time of tumor vasculature formation, before the infiltration of immune cells. Here, type I IFNs produced by endothelial cells is necessary for the infiltration and activation of cross-presenting DCs and CD8 T cells in the tumor microenvironment by the mechanisms described above. A likely source of endogenous STING ligands is tumor DNA or tumor-derived cGAMP, although it is unclear how they can spontaneously reach intracellular compartments of endothelial cells. Tumors may package DNA in apoptotic bodies and transfer it to endothelial cells (27), where it activates STING upon cGAS-mediated conversion to cGAMP (15, 16). Another possibility is that tumor cGAMP is directly transferred to endothelial cells via connexin-mediated channels, as this has been previously suggested (28).

Intratumoral STING activation by cGAMP and potentially other ligands may be an attractive candidate for antitumor therapy of metastatic melanoma patients. The efficacy of intratumoral cGAMP is strongly enhanced by combination with anti-CTLA4 and anti-PD1 therapy, the currently most potent immunotherapeutic approach in melanoma (29). Intratumoral cGAMP activation is also effective in the MC38 model of colon carcinoma, suggesting that other tumors can be treated by enforced intratumoral STING activation. These data were confirmed by the finding that local tumor radiotherapy of MC38 tumors can be enhanced by intratumoral cGAMP injection (18).

Our data identify a key role of the tumor vasculature arising in growing tumors in the shaping of CD8 T-cell–mediated antitumor immunity. This finding raises the question about the effect of antiangiogenic therapies (30), which are being developed and

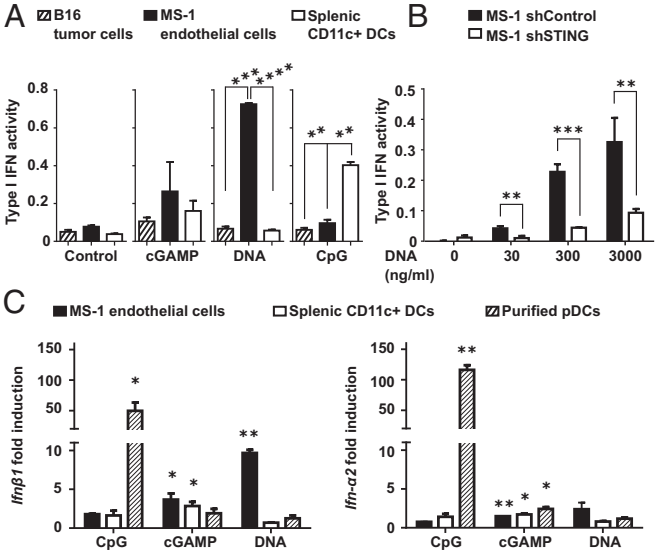

Fig. 5. Endothelial cells are specialized in producing large quantities of IFN-β in response to STING activation. (*A*) MS-1 endothelial cells, B16 tumor cells, and splenic CD11c+ DCs were stimulated overnight with cGAMP (5 μg/mL), tumor DNA (3 μg/mL), both complexed with Lipofectamine or with CpG-B (1 μg/mL). Type I IFN activity was measured in the culture supernatant. Data are expressed as mean ± SD and are representative of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by unpaired *t* test. (*B*) STING-deficient (shSTING) or control (shControl) MS-1 endothelial cells were stimulated overnight with increasing concentrations of tumor DNA complexed with Lipofectamine. Type I IFN activity was measured in the culture supernatant. Data are expressed as mean ± SD of two independent experiments. $^{**}P < 0.01$, $^{***}P < 0.005$ by unpaired *t* test. (*C*) MS-1 endothelial cells, spleen CD11c+ DCs, or purified spleen pDCs were activated for 5 h as described in *A*. *Ifn-β*1 and *Ifn-α*2 gene expression were determined by quantitative real-time PCR. Data are shown as fold induction over nonstimulated cells. $^{*}P < 0.05$, $^{**}P < 0.01$, by unpaired *t* test.

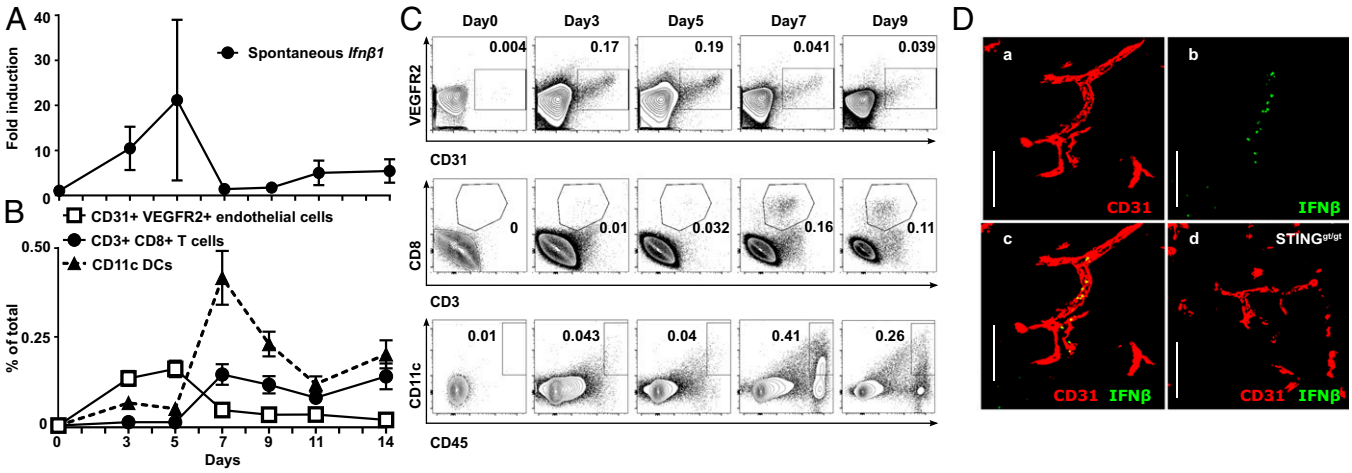

Fig. 6. Endothelial cells are the principal IFN-β producers in response to spontaneous STING activation in tumors. B16F10 melanoma cells were implanted into the flank of WT or STING$^{gt/gt}$ mice. (*A*) Time course analysis of spontaneous *Ifn-β1* mRNA expression in tumors after implantation in WT mice. Data represent the mean ± SEM of at least four independent mice. (*B*) Detection by flow cytometry of endothelial cells (VEGFR2+ CD31+ gated on CD45lo/neg), CD8 T cells (CD3+ CD8+), and DCs (CD45+ CD11c+) in single cell suspensions derived from tumors over time after their implantation. Data are expressed as mean ± SD of at least four independent mice. (*C*) Representative flow cytometry plot is shown. (*D*) Detection of spontaneous IFN-β production in tumors. Data show representative confocal microscopy images of 5-d-old tumors harvested from WT (*a*–*c*) or STING$^{gt/gt}$ mice (*d*) and stained for IFN-β and CD31. (Scale bar, 100 μm.)

considered to be used in combination with immune-based therapies, on antitumor immunity.

In conclusion, our study identifies STING to be the central molecular driver of spontaneous antitumor immune responses that can be exploited to generate potent antitumor immunity. We also identify endothelial cells as the principal cellular target of STING activation, uncovering an unexpected role of the tumor vasculature in the control of tumor growth via the initiation of antitumor immunity in melanoma.

Materials and Methods

Study Approval. These experiments were approved by the Institutional Animal Care and Use Committee of the University of Lausanne.

Tumor Model and Treatment. Mice were injected s.c. with 5×10^5 B16 melanoma cells. At 5 d after implantation, 10 μg of cGAMP complexed with Lipofectamine 2000 was injected into engrafted tumors. Tumor size was measured every 2 d using a caliper. Materials and methods are described in greater detail in *Supporting Information*.

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