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STING-Mediated DNA Sensing Promotes Antitumor and Autoimmune Responses to Dying Cells

Jared Klarquist,*† Cassandra M. Hennies,*† Maria A. Lehn,* Rachel A. Reboulet,* Sonia Feau,‡ and Edith M. Janssen*

Adaptive immune responses to Ags released by dying cells play a critical role in the development of autoimmunity, allograft rejection, and spontaneous as well as therapy-induced tumor rejection. Although cell death in these situations is considered sterile, various reports have implicated type I IFNs as drivers of the ensuing adaptive immune response to cell-associated Ags. However, the mechanisms that underpin this type I IFN production are poorly defined. In this article, we show that dendritic cells (DCs) can uptake and sense nuclear DNA-associated entities released by dying cells to induce type I IFN. Remarkably, this molecular pathway requires STING, but not TLR or NLR function, and results in the activation of IRF3 in a TBK1-dependent manner. DCs are shown to depend on STING function in vivo to efficiently prime IFN-dependent CD8+ T cell responses to tumor Ags. Furthermore, loss of STING activity in DCs impairs the generation of follicular Th cells and plasma cells, as well as anti-nuclear Abs, in an inducible model of systemic lupus erythematosus. These findings suggest that the STING pathway could be manipulated to enable the rational design of immunotherapies that enhance or diminish antitumor and autoimmune responses, respectively. The Journal of Immunology, 2014, 193: 6124–6134.

The immune system carefully balances its response to dead and dying cells to maintain homeostasis and prevent the development of autoimmunity. Although uptake and clearance of dying cells is generally considered a tolerogenic process, the existence of immunogenic cell death has been well described (1). Depending on the nature of the cell death, dying cells can emit damage-associated molecular patterns (DAMPs) (2) that act as danger signals and increase a dying cell’s immunogenicity. Many of these DAMPs seem to use the sensing and signaling pathways that are normally associated with the recognition and elimination of pathogens. Given the importance of immune responses to cell-associated Ags in autoimmunity, allograft rejection, and tumor rejection, the identification of these DAMPs, their cognate sensors, and their proinflammatory sequelae have become topics of intense research.

Ample studies have implicated type I IFNs in the development or progression of immune responses to self-Ags in autoimmune diseases such as rheumatoid arthritis, type I diabetes mellitus, Sjögren syndrome, and systemic lupus erythematosus (SLE) (3). However, type I IFNs were only recently identified as a crucial mediator in the priming of CD8+ T cells to cell-associated Ags in cancer and cancer treatments. Mice that lack type I IFN sensing, either by genetic IFN receptor (IFNAR) deletion or treatment with blocking Ab, develop more chemically induced tumors and show poorer rejection of transplanted immunogenic tumors than wild-type (WT) mice, highlighting the requirement for type I IFN in spontaneous tumor rejection (4, 5). Additional studies showed that the spontaneous induction of tumor-specific CD8+ T cells in tumor-bearing mice was predominantly mediated by type I IFN sensing in dendritic cells (DCs) (6, 7). A similar role for type I IFN was seen in therapy-induced tumor elimination. Burnette et al. (8) and Kang et al. (9) showed increased intratumoral production of type I IFN upon ablative radiotherapy or chemotherapy. The ablative effect of the therapy was associated with enhanced (cross)priming capacity of tumor-infiltrating DCs and could be abolished by eliminating IFNAR from the hematopoietic compartment. Our previous work, using tumor cell therapy in vaccination and therapeutic settings, showed a comparable dependency of type I IFN in the induction of protective antitumor CD8+ T cell responses (10–12).

Although the general immunostimulatory effects of type I IFN on DCs are well studied, little is known on the cellular source of type I IFN, the type I IFN–inducing ligand, and the receptor–signaling pathways involved in its induction upon the sensing and clearance of dying cells. Our previous work indicated that DCs can produce type I IFN upon phagocytosis of dying cells (10–12). Importantly, DCs from MyD88−/−/TRIF−/− mice showed normal type I IFN production upon phagocytosis of dying cells, and type I IFN–dependent CD8+ T cell priming to tumor cell vaccines was comparable in WT and MyD88−/−/TRIF−/− mice, indicating that the type I IFN induction requires an unidentified TLR-independent sensing pathway (11).

Using various murine cancer and tumor cell vaccination models and in vitro approaches, we show that the type I IFN production
upon sensing of dying cells is not only TLR independent, but also RLR independent, and requires STING/IRF3–mediated sensing of apoptotic cell–derived nuclear DNA structures by DCs. The ensuing type I IFN production enhances DC functionality in an autocrine manner, resulting in the increased clonal expansion, polyfunctionality, and memory formation of tumor-specific CD8+ T cells. Importantly, the role of the STING/IRF3/IFNAR nexus was not limited to CD8+ T cell priming or tumor models; elimination of STING or IFNAR significantly impacted the development of CD4+ follicular T cells (ThF), plasma cells, and anti-nuclear Abs in an inducible model of SLE. Collectively, our results demonstrate that STING/IRF3 sensing of nuclear DNA–derived structures by DCs broadly drives the priming of adaptive immune responses to dying cells.

Materials and Methods

Mice, cells lines, and peptides

Mice were maintained under specific pathogen-free conditions in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International. C57BL/6J, B6.PL-Thy1a2/CyJ (B6/CD90.1), B6.SJL-Peyer’s ileum (B6/CD45.1), CD11c–DTR, and B6(Cy)-H2-Ab1bm12/KiEg1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and H2-Kb+/– mice from Harlan (Indianapolis, IN). IFNAR–/–, MyD88–/–/TRIF–/–, CD11c–DTR–/–/IFNAR–/–, and Act-mOVA/H2-Kb+/– mice were bred in our facility. IRF3–/– and IRF7–/– mice were a gift from Dr. K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). STING+/–/mice a gift from Dr. R. Vance (University of Berkeley, Berkeley, CA), and IPS-1–/– mice a gift from Dr. J. Tschopp (University of Lausanne, Lausanne, Switzerland).

Mixed bone marrow (BM) chimeric mice were generated using donors on different congenic backgrounds in 1:1 ratios. All BM chimeric mice were rested for 12 wk before experiments were started. B16F10, B16-OVA (B16-OVA), EL-4mOV A, MEC.B7.SigOV A, MICE.B7.SigOVA, TAP-sufficient and -deficient MEFs expressing the human adenosine type 5 early region 1 (Ad5SEI-TAKO, 11.2), and ISRE-L929 IFN reporter cells have been described previously (13–16). Peptides OVA257–264 (SIINFEKL), E1B192–200 (VNRNCYCL), TRP-2100–108 (SVYDFFVWL), and LCMV GP33–41 (KAVYNFATC) were obtained from A&A Laboratories (San Diego, CA).

Cell isolations

T cells, B cells, Mph, and DCs were sorted by flow cytometry using markers TCRβ, CD4, CD8, CD11c, CD11b, CD19, CD45R, and MHC class II as described previously (12). Purity of sorted cells was generally >98% and viability was >97% as determined by 7-AAD staining.

Erythrocytes, normoblasts, reticulocytes, and RBCs were generated using a mouse-adapted protocol for the long-term ex vivo erythroid differentiation of cultures of human CD34 cells or mouse BM cells as described by Gardner et al. (17) and Kontstantinidis et al. (18). In brief, low-density BM cells were cultured in erythroblast growth medium (StemPro-34 with 2.6% StemPro-34 supplement; Invitrogen), 20% B1T 9500 (Stemcell Technologies), 900 ng/ml erythroblast growth medium (StemPro-34 with 2.6% StemPro-34 supplement; Invitrogen), 20% BIT 9500 (Stemcell Technologies), 900 ng/ml erythroblast growth medium (StemPro-34 with 2.6% StemPro-34 supplement; Invitrogen), 20% BIT 9500 (Stemcell Technologies), and 20% BF 9500 (Stemcell Technologies). Cells were cultured in a 1:3 ratio with irradiated cells (1500–3000 rad). Parallel experiments were performed with UV irradiation (120–240 mJ/cm2), Fax cross-linking (1–20 µg/ml), or etoposide treatment (6h, 1–10 µM) (11). When enucleated cells were compared with nucleated cells, all cells were gamma-irradiated and treated with thrombopoietin-1 to upregulate phosphorylase kinase on the membrane (19). After 20 h, type I IFN in the supernatant was determined by ISRE-L929 acidification studies, diphenyletheniodionium (10 µM), Con A (1 nM), or chloroquine (50 µM) were added at the start of the culture (12, 20). Parallel cultures using CpG and LPS were used as controls.

In vitro and ex vivo type I IFN analyses were performed by quantitative real-time PCR using SYBR Green and primers for β-actin (forward, 5′-TTGCTGACAGAGTGAGCAAG-3′; reverse, 5′-GACTTTGCTTGCAGAGGAGG-3′) and pan IFN-α (forward, 5′-TCTGATGACAGGTTGGGG-3′; reverse, 5′-GGGCTTCCAGACTTCTGCTG-3′). Samples were treated with DNase to eliminate genomic DNA contaminations. Gene expression was analyzed using the relative standard curve method and was normalized to Gapdh and β-actin expression.

Flow cytometry studies. All mice were incubated with CellTrace Violet (VT)–labeled irradiated splenocytes (Molecular Probes). After 3 and 16 h, DCs were stained with Abs to CD11c, CD11b, CD8α, nuclear dye Draq5, and fixable live/dead staining, and analyzed by ImageStream (Amnis, Seattle, WA). At least 10,000 live events were acquired, and the number and size of phagocytosed particles were determined using the spot counting and spot size features after tight masking on the bright-field image to exclude membrane-associated extracellular particles for each condition as shown before (12, 21).

To determine T cell activating capacity, DCs were irradiated OVA-Kb+/– cells for 4 h, after which CFSE-labeled purified OT-I–CD45.1 CD8+ T cells were added as described previously (11). OT-I cell proliferation and survival were determined after 7 h by analysis of CFSE dilution together with staining for CD8α, Vε2, CD45.1, and 7-AAD.

DC signaling studies

Imune coperditions. Purified DCs were irradiated with IRF3– or STING-deficient splenocytes. At different time points, DCs were sorted, lysed, precipitated using agarose-bound Abs to STING (3337; Cell Signaling) or IRF3 (D83B9; Cell Signaling), and probed for p-IRF3 (4D4G; Cell Signaling), TBK-1 (72B587; Novus Biologicals), p-STAT1 (D52C2; Cell Signaling), STAT6 (9362; Cell Signaling), and IPS-1 (77275; Novus Biologicals).

In parallel, VT–labeled irradiated IRF3–/– spleenocytes were cultured with WT and STING–/– DCs, and analyzed by flow cytometry and Amnis ImageStream upon surface staining with 2D11c–Pacific Blue and intracellular staining with Draq5 and p-IRF3 (anti-pS386, 4D4G) combined with anti-rabbit IgG Alexa Fluor 488. Correlations between intensity of phagocytosed material and p-IRF3 localization in the nucleus were determined using ImageStream by gating on CD11c+ cells followed by tight masking on the cells to discriminate internalized from bound particles followed by tight masking on the nucleus to determine colocalization of the p-IRF3 staining in the nucleus (12).

In vivo models

Immunizations. Mice were injected s.c. or i.p. with irradiated cells (OVA-Kb+/– spleenocytes, 106–107; B16-OVA, B16/F10, and SE1-TAKO, 1–5 × 106). Alternatively, mice received i.v. 2 × 106 purified DCs that had been pulsed with OVA257–264 or exposed to irradiated cells in vitro (10–12, 21). For depletion studies, CD11c–DTR mice and mixed BM chimeric mice were treated 24 h before and 24 h after immunization with 4 mg/dipytheria toxoid i.p.

Tumor models. For the cryoablation model, mice were s.c. injected with 4 × 105 B16-OVA or B16/F10 in PBS. Ten days later, tumors (7–10 mm) were cryoablated using Verruca-Freeze armed with a 6-mm probe (Brymill Cryogenic Systems) (22) for 3 × 25-s cycles. To test long-term tumor protection, we challenged the mice with 3 × 105 B16-OVA or B16/F10 40 d after the ablation of the primary tumor and monitored for tumor growth. In the EL-4–mOVA challenge model, mice were immunized with 104 irradiated OVA-Kb+/– spleenocytes as described earlier and 40 d later challenged with 105 EL-4–mOVA cells s.c.

SLE model. In the SLE model (23), mice of indicated genetic backgrounds received 3 × 105 B6C3H-H2-Ab1bm12/KiEg1 (bm12) spleenocytes i.p. Splenic B and T cell composition B was analyzed by flow after 2 wk. Serum levels of anti-dsDNA IgG, IgG1, and IgG2a were determined by ELISA as previously described (24).

T cell analysis

Unless stated differently, analyses were performed 7 d after immunization. CD8+ T cells were enumerated in spleens and draining lymph nodes using OVA257–264-Kb tetramers (Beckman Coulter) or E1B192–200-D8 decamers (Immudex) together with staining for CD8α, CD4, and 7-AAD. In parallel, cytokine production and polyfunctionality was determined directly ex vivo by intracellular cytokine staining after a 5-h stimulation with cognate peptide in the presence of brefeldin A as described previously (11, 16). Samples were collected on an LSR flow cytometer with Diva software (BD Pharmingen), and data were analyzed with FlowJo software (Tree Star). In B16/F10 studies, responses to TRP-2100–108 were determined by ELISPOT directly ex vivo.
Capacity for secondary expansion in vitro was determined by stimulating the splenocytes or purified CD8⁺ T cells on irradiated MEC.B7.SigOVA (OVA-specific) or E1.2 cells (E1B specific) for 6 d and dividing the absolute number of Ag-specific CD8⁺ T cells at the beginning of the culture by the absolute number of Ag-specific CD8⁺ T cells at the end of the culture as described previously (16, 25). In vivo secondary expansion was determined by reinjecting the mice with a 10-fold higher number of irradiated cells than used during immunization. Four days after the secondary challenge, the frequency of Ag-specific CD8⁺ T cells was compared with nonchallenged immunized mice (16).

Statistical analyses

Data were analyzed using Prism software (GraphPad Software). Unless stated otherwise, the data are expressed as means ± SEM. Survival responses were analyzed by Kaplan–Meier using a log-rank test. All other data were evaluated using a two-way ANOVA followed by a Dunnett’s test. A p value <0.05 was considered statistically significant.

Results

Protective antitumor immunity requires type I IFN sensing by DCs

We and others recently showed that type I IFN sensing is critical for the induction of protective antitumor responses in various tumor models in both vaccination and therapeutic settings (6–8, 22). Cryoablation of B16-OVA tumors in WT mice resulted in effective priming of OVA257-264-specific CD8⁺ T cells that provided protective immunity upon subsequent B16-OVA challenge. In contrast, IFNAR⁻⁻ mice failed to induce adequate OVA257-264-specific CD8⁺ T responses and succumbed upon tumor rechallenge (Fig. 1A, 1B). Similarly, immunization with gamma-irradiated, OVA-expressing K⁺⁻⁻ splenocytes (OVA-K⁺⁻⁻) induced a significantly more robust OVA257-264-specific CD8⁺ T cell response

**FIGURE 1.** Protective CD8⁺ T cell induction requires IFNAR on DCs. (A) WT and IFNAR⁻⁻ mice were s.c. injected with B16-OVA tumor cells, and palpable tumors were cryoablated 10 d later. Cryoablated and naive mice were s.c. challenged with B16-OVA 40 d later and survival was monitored (n = 8–10/group). (B) OVA257-264-specific CD8⁺ T cell frequency in the spleen 7 d after cryoablation. (C) Mice were immunized with irradiated OVA-K⁺⁻⁻ or OVA-K⁺⁻⁻ splenocytes and 40 d later s.c. challenged with EL-4-mOVA cells s.c. (D) Frequency of splenic OVA257-264-specific CD8⁺ T cells 7 d after immunization as determined by intracellular cytokine staining for IFN-γ after peptide restimulation and by OVA257-264-K⁺⁻⁻tetramer staining. (E) Fold expansion of OVA257-264-specific CD8⁺ T cells upon stimulation with OVA257-264-expressing cells in vitro. Data of representative experiments (out of three to five) are shown (mean ± SEM, n = 8–10/group, *p < 0.05).
in WT mice than in IFNAR−/− mice (Fig. 1C, 1D). The OVA257–264–specific CD8+ T cells from WT mice, but not IFNAR−/− mice, underwent expansion upon secondary encounter with Ag in vitro and in vivo, and protected mice from EL-4-mOVA challenge in vivo (Fig. 1E, Supplemental Fig. 1A). Importantly, this response was not restricted to OVA or the selected pathways of cell death. Similar results were found when the parental line B16/F10 was used and the response to self-Ag TRP-2 was probed (Supplemental Fig. 1B, 1C). In addition, direct immunization with gamma-irradiated, UV-irradiated, Fas-cross-linked, or etoposide-treated cells showed significantly decreased CD8+ T cell responses in IFNAR−−/− mice, illustrating a central role for type I IFN in CD8+ T cell priming to cell-associated Ags in various scenarios of cell death (Fig. 2).

Because nearly all cells express IFNAR, we first used BM chimeras to identify which cells required type I IFN sensing in the CD8+ T cell response to dying cells. BM chimeric mice (WT→IFNAR−−/−, WT→WT, IFNAR−−/−→WT, IFNAR−−/−→IFNAR−−/−) demonstrated that type I IFN needed to be sensed by the hematopoietic compartment (Fig. 3A, 3B). Additional studies with WT/IFNAR−−/→WT mixed BM chimeric mice indicated that the diminished CD8+ T cell response could not be attributed to the lack of IFNAR on the CD8+ T cells because IFNAR−−/−→CD8+ T cells showed only a marginal decrease in clonal expansion (Fig. 3C). Given the important role of DCs in cross-presentation of cell-associated Ags, we next examined the role of type I IFN sensing on DCs. Mixed BM chimeras were generated in which IFNAR−−/− recipients received a combination of WT-CD11c-DTR and IFNAR−−/−CD45.1 BM or IFNAR−−/−CD11c-DTR and WT/CD45.1 BM. In this model, the administration of diphtheria toxin and interleukin-12p40 (DT)-treated, CD11c-DTR mice upon s.c. immunization with actmOVA-Kb−−/− or control peptide GP33–41 (white bar, control peptide; black bar, specific peptide). Data in all experiments are expressed as mean ± SEM with n = 5. *p < 0.05.

**FIGURE 2.** Impaired CD8+ T cell priming in IFNAR−−/− mice is independent of the method of cell death or cell type. Frequency of splenic Ag-specific CD8+ T cells in WT and IFNAR−−/− mice 7 d after s.c. immunization with actmOVA-Kb−−/− splenocytes (which were gamma irradiated [1500 rad], UV irradiated [120 mJ/cm2], or treated with FAS cross-linking Ab [Jo-1, 20 μg/ml; 4 h, 37°C]), 5E1-TAKO cells (3000 rad, 240 mJ/cm2), or treated with etoposide [10 μM]), or B16-OVA or B16/F10 cells (3000 rad or 240 mJ/cm2). Ag-specific T cell frequencies were determined by intracellular cytokine staining upon incubation with OVA257–264, E1β192–200, TRP-2180–188, or control peptide GP33–41 (white bar, control peptide; black bar, specific peptide). Data in all experiments are expressed as mean ± SEM with n = 5. *p < 0.05.
DCs, indicating that the IFN induction was TLR, RIG-I, and Mda5 independent. In contrast, significant reductions in type I IFN production were seen with DCs deficient in STING and the transcription factor IRF3 even though their subset compositions, maturation status, and phagocytic capacity were similar to WT DCs (Fig. 5A, 5B, and data not shown). Immunoprecipitation and Western blotting studies indicated rapid IRF3 phosphorylation in WT DCs and a significant reduction and delay in IRF3 phosphorylation in STING−/−DCs upon exposure to dying cells (Fig. 5C, 5D). To determine whether the magnitude of the nuclear

**FIGURE 3.** Selective requirement for type I IFN sensing in the DCs. (A) Bone-marrow chimeric mice (WT→IFNAR−/−, WT→WT, IFNAR−/−→WT, IFNAR−/−→IFNAR−/−) were immunized i.p. with irradiated 5E1-TAKO cells and the frequency of E1B 192–200–specific CD8+ T cells in the spleens was determined 7 d later (white bar, control peptide; black bar, E1B192–200 peptide). (B) The effect on memory formation using these chimeras was determined by measuring Ag-specific T cell expansion ex vivo. Secondary expansion of E1B 192–200–specific CD8+ T cells was calculated by dividing the absolute number of E1B 192–200–specific CD8+ T cells at the end of a 6 d culture by the absolute number at the start of the culture. (C) WT/CD90.1 mice were irradiated and reconstituted with WT, IFNAR−/− or a 1:1 ratio of WT/CD45.1:IFNAR−/− (CD45.2) bone marrow. Mice were i.p. immunized with irradiated 5E1-TAKO cells and the frequency of splenic E1B 192–200–specific CD8+ T cells in each graft was determined 7 d later. (D) WTCD11cDTR:IFNAR−/− and WT:IFNAR−/−CD11cDTR mixed bone-marrow chimeras were treated with DT or vehicle and immunized with irradiated 5E1-TAKO cells. The frequency of E1B 192–200–specific CD8+ T cells was determined 7 d later. Data of representative experiments (out of three to five) are shown (mean ± SEM, n = 5/group, *p < 0.05).

**FIGURE 4.** Type I IFN production by DCs in vitro and in vivo. Total and subset-depleted splenocytes (A) or purified cell populations (B) from spleens of naive WT mice were cultured with irradiated OVA-Kb2/2 splenocytes, and type I IFN in the supernatant was determined 20 h later (black bars, irradiated cells; white bars, no cells). Data are expressed as mean ± SEM with n = 4. (C) Splenocytes from CD11c-DTR mice (treated with PBS or DT 24 h prior) were isolated and cultured with irradiated OVA-Kb2/2 splenocytes. Type I IFN in the supernatant was determined 20 h later. (D) CD11c-DTR mice were treated with vehicle or DT and 24 h later s.c. injected with irradiated cells. Type I IFNa mRNA in draining lymph nodes was assessed 6–8 h later. Representative data of one experiment (out of three to four) are shown (mean ± SEM, n = 5, *p < 0.05).
p-IRF3 signal was associated with the frequency and size of phagocytosed particles, we incubated WT and STING−/− DCs with VT-labeled, irradiated splenocytes. Although STING−/− DCs displayed similar uptake of VT material as WT DCs, they exhibited significantly reduced nuclear p-IRF3 staining, consistent with their limited IFN production (Fig. 5E, 5F, Supplemental Fig. 2A). Using UV irradiation, Fas cross-linking, or etoposide to induce cell death yielded similar outcomes, indicating that the STING/IRF3 pathway was the dominant IFN-inducing pathway in various forms of sterile cell death (Supplemental Fig. 2B).

**Nuclear DNA-derived structures induce type I IFN production by DCs**

We next set out to determine the ligand upstream of STING that was responsible for inducing type I IFN. STING facilitates immune responses to various nucleotide structures, including cytosolic dsDNA, and in some cases dsRNA (26–28). Because dsRNA sensing uses the RIG-I/IPS-1 pathway and IPS1−/− DCs have normal type I IFN production, it is likely that the IFN-inducing species released by dying cells is a DNA-based and not an RNA-based entity. Indeed, addition of DNases, but not RNases, to WT DC/irradiated cell cocultures significantly reduced type I IFN production without affecting uptake of cellular material or responses to non-nucleic TLR ligands (Fig. 6A, Supplemental Fig. 2C, 2D). To more rigorously address the role of DNA complexes in the type I IFN induction, we exploited the process of erythropoiesis where RBC precursors sequentially lose their nuclei, mitochondria, and ribosomes. Timed RBC cultures were sorted, irradiated and treated with thrombospondin-1 (irr/TSP) to induce comparable phosphatidylserine expression on the membrane and facilitate an “apoptotic phenotype” in the non-nucleated cells (Fig. 6B). ImageStream analysis showed comparable uptake of the irr/TSP cell subsets by DCs (Fig. 6C). Nucleated irr/TSP

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**FIGURE 5.** Type I IFN induction requires the STING/IRF3 pathway. (A) Type I IFN production by purified DCs from indicated strains upon 20-h culture with irradiated splenocytes. (B) Purified DCs from indicated strains were cultured at 4˚C (white bars) and 37˚C (black bars) with VT-labeled irradiated splenocytes. Uptake of VT materials was determined 6 h later by flow cytometry. Data are expressed as percentage of DCs that contain VT. Representative data of one experiment (out of three to four) are shown (mean ± SEM, n = 4). (C and D) p-IRF3 kinetics in WT and STING−/− DCs upon incubation with irradiated IRF3−/− splenocytes. (E) ImageStream images of p-IRF3 nuclear translocation in WT DCs 8 h after incubation with VT-labeled irradiated IRF3−/− cells. Original magnification ×60. (F) ImageStream analysis of nuclear p-IRF3 intensity and intensity of cytosolic phagocytosed material in WT and STING−/− DCs. Tight masking on the cytosol was done to discriminate between bound and internalized cellular material. At least three experiments were performed with 800–1000 analyzed cells/condition. *p < 0.05
erythroblasts readily induced type I IFN in DCs, whereas enucleated reticulocytes and RBCs failed to do so (Fig. 6D). These data indicate that the IFN-inducing species is nuclear DNA derived.

**DC-intrinsic STING regulates CD8+ T cell responses to dying cells**

Given the importance of STING-mediated DNA sensing in the type I IFN production, we next assessed the relative contribution of STING in the priming of CD8+ T cells to dying cell-associated Ags. WT, MyD88−/−/Trif−/−, IPS−/−, and IRF7−/− mice showed comparable CD8+ T cell priming as determined by tetramer staining, intracellular cytokine staining, and capacity for secondary expansion upon immunization with irradiated 5E1-TAKO cells (Fig. 7A, 7B). In contrast, IFNAR−/−, IRF3−/−, and STING−/− mice showed significantly reduced CD8+ T cell priming (Fig. 7C, 7D). Moreover, the Ag-specific IFNAR−/−, IRF3−/−, and STING−/− CD8+ T cells displayed less cytokine polyfunctionality and impaired capacity for secondary expansion (Fig. 7E). The defect in CD8+ T priming was fully DC regulated as similar results on CD8+ T cell clonal burst, secondary expansion, and cytokine polyfunctionality were seen when purified IFNAR−/−, IRF3−/−, and STING−/− DCs were exposed to irradiated cells in vitro and transferred into WT recipients (Fig. 7F–H). The priming defect of the IFNAR−/−, IRF3−/−, and STING−/− DCs could not be attributed to a decrease in overall DC functionality as peptide-pulsed IFNAR−/−, IRF3−/−, and STING−/− DCs induced comparable CD8+ T cell responses as WT DCs upon transfer in WT recipients (Fig. 7I). Together, these data demonstrate the requirement for STING, IRF3, and IFNAR in DCs in the cross-priming of CD8+ T cells to cell-associated Ags.

**STING regulates CD4+ T cell and B cell responses in the bm12 SLE model**

To assess whether STING has a broader role in the priming of adaptive immune responses to dying cells, we assessed the induction of CD4+ T cell and B cell responses in the bm12-cGVHD model where H-2b B6 hosts develop lupus-like disease upon transfer of B6.C-H2bm12 CD4+ T cells (29). Both IFNAR−/− and STING−/− mice developed considerably less activated CD4+ T cells and Th than WT recipients upon transfer of bm12 CD4+ T cells (Fig. 8A, 8B). Moreover, both IFNAR−/− and STING−/− mice developed considerably less activated B cells, plasma cells, and pathogenic anti-dsDNA IgG2a Abs than WT recipients (Fig. 8A, 8C, 8D). Given that the transferred bm12-CD4+ T cells were IFNAR and STING sufficient, these data further support the role for Ag-presenting, cell-intrinsic STING and IFNAR in the induction of adaptive immune responses to dying cell-derived Ags.

**Discussion**

Type I IFNs have been implicated as the upstream events precipitating autoimmune disease and a prerequisite for effective antitumor radiotherapy. In this study, we identify DC sensing of cell-derived nuclear DNA entities via the STING/IRF3 pathway as a key component in the early type I IFN response to dying cells.

Dying cells can emit a plethora of structurally distinct DAMPs, and it is likely that the molecular pathways involved in the sensing of these DAMPs are equally diverse. Although many DAMPs can contribute to the final adaptive immune response to cell-associated Ags, our data identified type I IFN as the dominant proinflammatory factor and STING/IRF3 signaling as the principle pathway in the initiation of the immune responses to cell-associated Ags.
Ags in our tumor models, as well as the autoimmune responses in our SLE model. Although all nucleated cells can sense type I IFN, and type I IFN has been shown to directly act on T cells, our data indicate that the early STING/IRF3-mediated type I IFN predominantly acts on DCs. Transfer of IFNAR2/2 DCs (pulsed with irradiated cells) into WT recipients resulted in similar deficiencies in CD8+ T cell expansion, functionality, and memory formation as the direct immunization of IFNAR2/2 mice. Moreover, immunization of WT/IFNAR2/2 mixed BM chimeric mice did not show any significant difference in CD8+ T cell clonal expansion or polyfunctionality between the WT and IFNAR2/2 grafts. Importantly, transfer of STING+/+ or IRF3+/+ DCs into WT recipients resulted in similar defects in CD8+ T cell responses as the transfer of IFNAR2/2 DCs. Likewise, STING2/2 recipients showed identical reduction in Tfh and plasma cell formation as IFNAR2/2 recipients in our SLE model. Together with the observation that STING−/− and IRF3−/− DCs have significantly reduced type I IFN induction upon phagocytosis of dying cells, these data implicate that the STING/IRF3 pathway is the critical component in the type I IFN–dependent T cell priming to cell-associated Ags.

It has been suggested that different types of death may induce different DAMPs. We observed comparable type I IFN induction and STING/IRF3 engagement in DCs upon phagocytosis of cells treated with gamma irradiation, UV irradiation, Fas–cross-linking Ab, or etoposide, suggesting that these different types of cell death generated a similar nuclear DNA-derived DAMP (Supplemental Fig. 2B and data not shown). It is also likely that similar nuclear DNA-associated DAMPs become available in vivo as type I IFN induction, and type I IFN–dependent CD8+ T cell priming was readily observed in WT and MyD88/Trif-deficient mice, but sig-

**FIGURE 7.** STING regulates the CD8+ T cell responses to dying cells in vivo. (A) Mice of indicated strains were immunized with irradiated 5E1-TAKO cells, and the frequency of splenic E1B192–200–specific CD8+ T cells was determined 7 d later (white bar, control peptide; black bar, E1B192–200 peptide). (B) Fold expansion of E1B192–200–specific CD8+ T cells from indicated mouse strains upon culture with E1B192–200–expressing feeder cells in vitro. (C) E1B192–200–specific CD8+ T cell frequency in indicated mouse strains 7 d after immunization with irradiated 5E1-TAKO cells. (D) Secondary expansion of E1B192–200–specific CD8+ T cells in vitro. (E) Ex vivo polyfunctionality of E1B192–200–specific CD8+ T cells from (C) as determined by flow cytometry. (F) Frequency of E1B192–200–specific CD8+ T cells in WT mice 7 d after transfer of indicated DCs pulsed with irradiated 5E1-TAKO cells in vitro. (G) Secondary expansion of E1B192–200–specific CD8+ T cells primed by indicated DCs. (H) Ex vivo polyfunctionality of E1B192–200–specific CD8+ T cells primed by indicated DCs. (I) DCs were purified from WT, IFNAR2/2, and STING2/2 mice and exposed to irradiated 5E1-TAKO cells or pulsed with 1 μM E1B192–200 peptide. DCs were repurified and 2 × 105 were injected into WT recipients. Seven days later, the frequency of splenic E1B192–200–specific CD8+ T cells was determined (white bar, control peptide; black bar, E1B192–200 peptide). Representative data of one experiment (out of three to four) are shown (mean ± SEM, n = 5–7, *p < 0.05).
significantly reduced in STING<sup>−/−</sup> and IRF3<sup>−/−</sup> mice upon administration of dying cells (gamma, UV, or FAS treated) or upon in vivo tumor cryoablation (11, 22).

Importantly, the crucial role for type I IFN in the priming of protective adaptive antitumor responses is not restricted to situations where massive tumor cell death occurs, as is the case for radiotherapy, chemotherapy, and cryoablative tumor therapies. Recent publications indicate that spontaneous and limited tumor cell death in tumor-bearing mice also resulted in type I IFN–dependent protective immune responses. Fuertes et al. (7) and Diamond et al. (6) showed spontaneous antitumor CD8<sup>+</sup> T cell induction and tumor rejection in tumor-bearing mice that was critically dependent on type I IFN sensing by cross-priming DCs. In this light, it is interesting to note that STING<sup>−/−</sup> mice, like IFNAR<sup>−/−</sup> mice, develop significantly more lung metastases than WT mice upon i.v. injection of low numbers of untreated B16 melanoma cells, illustrating a role for the STING/IFNAR nexus in the antitumor response when cell death is limited (data not shown).

STING can facilitate innate responses to cytosolic bacterial cyclic dinucleotides (cyclic-di-GMP) (30, 31), dsDNA, and in some cases cytosolic dsRNA (26–28). Our data strongly suggest that the main type I IFN–inducing ligand is a nuclear DNA species. STING-mediated dsRNA sensing requires RNA with 5′-triphosphate groups in combination with the IPS-1/RIG-I pathway. The absence of IPS-1 recruitment to STING in WT DCs upon phagocytosis of cellular materials, as well as the normal type I IFN production and CD8<sup>+</sup> T cell responses in IPS1<sup>−/−</sup> mice, effectively argue against a role for dsRNA sensing in the STING/IFN phenotype. Our hypothesis that the type I IFN–inducing ligand is a DNA structure is strongly supported by our in vitro data that show significantly reduced type I IFN induction upon addition of DNases to the dying cell/DC coculture. Moreover, the use of enucleated cells dramatically reduced type I IFN production and cross-priming by WT DCs in vitro. Importantly, the latter experiment also suggested that mitochondrial or ribosomal nucleotide structures had no notable role in the type I IFN production as reticulocytes, enucleated but still containing mitochondria and ribosomes, failed to induce type I IFN.

Recent studies indicate direct binding of cyclic-di-GMP and cyclic-GMP-AMP (cGAMP) to STING but have not provided evidence for direct dsDNA–STING interactions (32, 33) suggesting the involvement of upstream DNA sensors. Over the last few years, several candidate sensors have been identified, including cGAMP synthase (cGAS), which has been shown to signal through STING via the production of cGAMP (34–37). DExD/H-box protein family member DDX41 and IFI16 (p204) have also been implicated as possible DNA sensors acting through STING, but their precise molecular interactions have not been fully elucidated (38, 39). Currently, these candidate DNA sensors...
are studied in in vitro systems where the DNA is directly delivered into the cytosol via transfection, transduction, or infection pathways. However, for the phagocytosed DNA-derived structures to be sensed by the STING pathway, either its key components should be recruited to the phagosome or the DNA-derived species should escape into the cytosol. Although STING can translocate from the endoplasmic reticulum to the Golgi and autophagosome-like compartments, we and others did not observe STING in phagosomes or phagolysosomes (data not shown) (40–44). However, phagosomal DNA sensing via STING could still be possible by phagosomal recruitment of p204 that has reported migratory capacity or cGAS that produces the highly mobile secondary messenger cGAMP (34, 36, 39). In contrast, phagosomal escape is a well-reported process in cross-presentation where protein structures escape into the cytosol to be processed for presentation in MHC class I (20, 45). The exact mechanism by which proteins escape into the cytosol is not known, but it is strongly associated with alkalization of the phagosome and prevention of phagosomal acidification (20, 45, 46). Consistent with the latter possibility, STING-mediated type I IFN production was strongly associated with inhibition of phagosomal acidification (45); in vitro treatments of DCs with agents that accelerated phagosomal acidification decreased type I IFN production, whereas alkalization or the delay of endosomal acidification significantly enhanced type I IFN production (Supplemental Fig. 3). Moreover, the deletion of TREX1 (DNase III, a DNA exonuclease with onset in infancy (SA VI), a syndrome characterized by a severe immunodeficiency, Central Nervous System dysfunction, and onset of systemic lupus erythematosus) or STING-/- background (48–51). Intriguingly, gain-of-function mutations in STING were recently associated with vasculopathy with onset in infancy (SAVI), a syndrome characterized by a severe cutaneous vasculopathy leading to extensive tissue loss and structural damage, with neonatal-onset systemic inflammation (52). Moreover, the deletion of TREX1 (DNase III, a DNA exonuclease with functions in DNA degradation) was shown to trigger type I IFN–associated inflammatory responses in a cGAS-dependent manner (53, 54). Furthermore, mutations in TREX1 have been associated with a variety of (multiorgan) inflammatory syndromes, including SLE, Sjögren syndrome, chilblain lupus, retinal vasculopathy, and Aicardi-Goutières syndrome (53–60).

Together, our data indicate that sensing of nuclear DNA–derived structures via the STING/IRF3 pathway in DCs is responsible for the early type I IFN induction upon phagocytosis of dying cells. This early type I IFN directly acts on the DCs and endows them with greater capacity to activate adaptive immune responses to cell-associated Ags. Given that type I IFNs have been implicated as the upstream events precipitating autoimmune disease and a prerequisite for effective antitumor therapy, further characterization of the STING pathway as sensor of dying cell–derived DNA could enable the rational design of new therapies to enhance antitumor responses and interfere with the development and progression of autoimmune diseases.

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Disclosures
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