STING Pathway Activation Stimulates Potent Immunity against Acute Myeloid Leukemia

Graphical Abstract

Highlights
- Unlike solid cancers, a type I IFN response is not triggered in AML-bearing hosts

- STING activation induces expression of IFN-β and other inflammatory cytokines

- STING activation promotes DC maturation and leukemia-specific T cell priming

- Enhanced immunity translates into prolonged survival in mice with AML

Authors
Emily Curran, Xiufen Chen, Leticia Corrales, ..., Priyanka Duttagupta, Marcin Kortylewski, Justin Kline

Correspondence
jkline@medicine.bsd.uchicago.edu

In Brief
Curran et al. demonstrate that, in contrast to solid cancers, a host type I IFN response is not triggered in leukemia-bearing hosts. However, induction of type I IFN and other inflammatory cytokines through STING pathway activation results in potent leukemia-specific immunity, culminating in prolonged survival of mice with AML.
STING Pathway Activation Stimulates Potent Immunity against Acute Myeloid Leukemia

Emily Curran, Xiufen Chen, Leticia Corrales, Douglas E. Kline, Thomas W. Dubensky, Jr., Priyanka Duttagupta, Marcin Kortylewski, and Justin Kline

INTRODUCTION

Anti-tumor T cell responses develop spontaneously in a fraction of cancer patients and the presence of tumor-infiltrating T cells has prognostic implications (Galon et al., 2006; Pagés et al., 2005; Mahmoud et al., 2011; Hwang et al., 2012). How the sterile tumor environment supports tumor-specific T cell priming has been of significant interest in recent years. Gene expression profiling of human melanomas has revealed a type I interferon (IFN) signature in tumors heavily infiltrated by CD8+ T cells (Gajewski, 2007), suggesting that type I IFN might be important for bridging innate and adaptive anti-tumor immune responses. Direct evidence in support of this hypothesis has come from animal models in which type I IFN signaling in host cells was necessary to promote spontaneous anti-tumor CD8+ T cell priming (Fuertes et al., 2011; Diamond et al., 2011). Furthermore, several groups have demonstrated that transplanted and carcinogen-induced solid tumors grow more rapidly in type I IFN receptor-deficient (Ifnar-/-) mice (Dunn et al., 2005; Fuertes et al., 2011). Collectively, these observations have established an essential role for type I IFN in generating adaptive immune responses against solid cancers.

The cancer cell-derived signals that induce host type I IFN production remained elusive until it was shown that tumor DNA triggered IFN-β production by dendritic cells (DCs) in vivo through activating the cytosolic DNA-sensing stimulator of IFN genes (STING) pathway (Woo et al., 2014). In the native STING pathway, cyclic dinucleotides known as cyclic GMP-AMPs (cGAMPs) are generated from cytosolic DNA by the enzyme cGAMP synthase (cGAS) (Sun et al., 2013; Wu et al., 2013). Upon cGAMP binding, the STING homodimer undergoes a significant conformational change and traffics from the endoplasmic reticulum to the Golgi, where it recruits tank-binding kinase 1 (TBK1), resulting in its phosphorylation, activation of IFN regulatory factor 3 (IRF3), and transcription of type I IFN (Ishikawa and Barber, 2008; Ishikawa et al., 2009). STING also activates the STAT6 and necrosis factor kappa B (NF-κB) pathways, inducing the expression of a number of inflammatory cytokines and chemokines, including CCL20, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) (Sun et al., 2013; Wu et al., 2013; Chen et al., 2011; Fu et al., 2015).

Recent work indicates that, in the setting of a localized tumor, spontaneous anti-tumor CD8+ T cell responses are abrogated in STING-deficient hosts, but they occur normally in mice deficient in other nucleic acid-sensing receptors (Woo et al., 2014), suggesting that tumor-derived DNA and STING are critical for generating adaptive anti-tumor immunity. However, in contrast to solid malignancies, hematological cancers, such as acute myeloid leukemia (AML), are typically disseminated at inception and lack classical draining lymph nodes. While it has been demonstrated that AML cells can be recognized by the host immune system (Ohminami et al., 2000), the mechanisms that regulate immunity and immune tolerance against this disease and, specifically, the roles of STING and type I IFN are relatively unknown. Interestingly, our recent work has implicated antigen-presenting cells (APCs), producers of type I IFN, in generating a unique T cell-tolerant state in AML-bearing animals (Zhang et al., 2013), suggesting that the host type I IFN response may not be
activated in this disease. If this is the case, strategies aimed at stimulating type I IFN production in AML-bearing hosts, such as through STING activation, might lead to effective adaptive immunity against leukemia-derived antigens.

Here we demonstrate that a host type I IFN response is not generated in mice with systemic AML. Further, the survival of leukemia-bearing mice is similar in the presence or absence of host type I IFN signaling and STING, in sharp contrast to what has been observed in solid tumor models (Fuertes et al., 2011; Diamond et al., 2011; Woo et al., 2014). However, administration of the STING agonist 5,6-demethylxanthenone-4-ace- tic acid (DMXAA) to animals with established AML induces type I IFN and TNF-α production, leads to APC maturation, and culminates in extremely potent activation of leukemia antigen-specific CD8+ T cells. DMXAA treatment significantly prolongs the survival of and, in some cases, cures mice with AML. Collectively, these results provide strong rationale for the therapeutic development of STING agonists as immunotherapy for AML.

RESULTS

Disseminated AML Fails to Induce a Host Type I IFN Response

Host type I IFN signaling is necessary for the generation of spontaneous CD8+ T cell responses against solid tumors (Fuertes et al., 2011; Diamond et al., 2011). To determine whether type I IFN was induced in mice with systemic leukemia, C1498 AML cells were inoculated intravenously (i.v.) into C57BL/6 mice and Ifnb expression was measured in bulk spleen cells. Ifnb expression was also analyzed in the tumor-draining lymph nodes (TDLNs) of mice given a localized (subcutaneous [s.c.]) C1498 challenge as a positive control (Fuertes et al., 2011). Ifnb mRNA levels were similarly low in spleen cells from leukemia-free and i.v. C1498 cell-challenged animals. In contrast, Ifnb expression could be readily detected in TDLN cells from s.c. C1498 cell-challenged mice, as expected (Figure 1A). To determine whether type I IFN signaling was important for the generation of functional immune responses to systemic AML, survival of wild-type and Ifnar−/− mice given an i.v. challenge with C1498 cells was compared, and it was found to be quite similar (Figure 1B). Conversely, and in agreement with published data, tumors derived from s.c.-implanted C1498 cells progressed more rapidly in Ifnar−/− compared to wild-type mice (Figure 1C). Collectively, these results indicate that a disseminated leukemia fails to stimulate a type I IFN response in the host.

The STING Agonist DMXAA Induces IFN-β, TNF-α, and IL-6 Expression In Vivo

Various STING agonists, including synthetic cyclic dinucleotides (CDNs) and DMXAA, have been used therapeutically with success, either when injected intra-tumorally or when administered as part of a localized cancer vaccine (Corrales et al., 2015; Fu et al., 2015). Because IFN-β was not induced in animals with systemic AML, it was of interest to determine if a systemically delivered STING agonist could generate a host type I IFN response sufficient to mediate control or rejection of AML. Following i.v. administration, DMXAA induced Ifnb expression in spleen cells in a STING-dependent manner, as demonstrated by lack of Ifnb expression in DMXAA-treated Tmem173−/− (STING-deficient) mice (Figure S1A). STING also activates NF-κB through a poorly understood mechanism. Consistent with this, enhanced Tnfa and Il6 expression levels were observed in the spleens of DMXAA-treated mice, also in a STING-dependent manner (Figures S1B and S1C). Serum levels of IFN-β, TNF-α, and IL-6 also were elevated following DMXAA treatment (Figures S1D–S1F).

DMXAA Treatment Activates Host APCs

Type I IFN has been shown to directly activate APCs (Montoja et al., 2002). Because DMXAA administration induced type I IFN, an in vivo effect on DCs and macrophages was observed in wild-type and Ifnar−/− mice. Using flow cytometry, we found that both CD8α+ and CD11c+ cells from DMXAA-treated mice were significantly more activated than in control mice (Figure S1G–S1I). These findings indicate that DMXAA triggers an immune response in vivo that is dependent on both STING and IFN-β.

Figure 1. Systemic AML Fails to Induce a Host Type I IFN Response

(A) C1498 cells (5 × 10^6) were inoculated i.v. or s.c. into C57BL/6 mice. Control mice received PBS i.v. Three days later, Ifnb expression in bulk spleen cells (i.v. C1498 or PBS mice) or in draining lymph node cells (s.c. C1498 mice) was analyzed by qRT-PCR and normalized to controls that received PBS i.v. Data are pooled from three independent experiments each with three mice per group and represented as mean ± SEM. (B) C57BL/6 and Ifnar−/− mice were challenged with 10^6 C1498 cells i.v. and survival was assessed. (C) C57BL/6 and Ifnar−/− mice received 10^6 C1498 cells s.c. and tumor volume was assessed over time. Data are pooled from two independent experiments, each with four to five mice per group, and represented as mean ± SEM.

n.s., not significant; **p < 0.01 and ***p < 0.001.
next investigated through an analysis of their cell surface expression of co-stimulatory molecules and major histocompatibility complex (MHC) class II molecules, as well as IL-12 production. Increased expression of CD80, CD86, and MHC class II was observed on DCs and, to a lesser extent, on macrophages from DMXAA-treated animals, again in a STING-dependent manner (Figures S2A and S2B). Furthermore, IL-12 production was 2- to 3-fold higher in DCs following DMXAA treatment. Macrophages did not produce any detectable IL-12 at baseline or following STING activation (Figures S2C and S2D).

Collectively, these data demonstrate that DMXAA treatment activates APCs, and DCs in particular, which may enhance their capability to stimulate adaptive anti-leukemia immune responses.

**STING Activation Stimulates Potent Leukemia-Specific CD8+ T Cell Responses**

Having shown that DMXAA treatment induces type I IFN expression and APC activation, its effect on leukemia-specific CD8+ T cell responses was next investigated. C57BL/6 mice were challenged i.v. with C1498.SIY cells, and subsequently they were treated with DMXAA or vehicle control. 1 week later, endogenous SIY-specific CD8+ T cell responses were analyzed in the spleen and bone marrow of leukemia-bearing animals. As shown in Figures 2A–2C, a striking expansion of SIY-specific CD8+ T cells occurred in DMXAA- versus vehicle control-treated mice. Importantly, the effect of DMXAA on the expansion of SIY-specific T cells required their exposure to cognate antigen, as no increase in the frequency or number of antigen-specific CD8+ T cells occurred in DMXAA-treated, leukemia-free animals (Figures 2A–2C). Antigen-specific CD8+ T cells in DMXAA-treated, leukemia-bearing animals were functional and produced IFN-γ following ex vivo re-stimulation (Figures 2D and 2E). In contrast, very few IFN-γ+ CD8+ T cells were generated in vehicle control-treated mice with AML (Figures 2D and 2E). Also important was the observation that DMXAA did not induce IFN-γ production by polyclonal CD8+ T cells in naive mice (Figures 2D and 2E). The effect of DMXAA on enhanced leukemia-specific CD8+ T cell priming was completely STING dependent, and it did not occur in leukemia-bearing Tmem173+/− hosts treated with DMXAA (data not shown). These data demonstrate that STING activation promotes the robust expansion of endogenous leukemia antigen-specific T cells.

To directly track the proliferation and expansion of AML-specific CD8+ T cells following STING activation, T cell receptor (TCR) transgenic (Tg) CD8+ 2C T cells, which specifically recognize the SIY antigen, were CTV labeled and adoptively transferred into C57BL/6 mice. C1498.SIY AML cells were then inoculated i.v. and DMXAA or vehicle control was administered. In leukemia-free mice, 2C T cells remained largely undivided, whether or not DMXAA was administered, indicating that STING activation did not stimulate leukemia-specific T cells in the absence of antigen (Figures S3A and S3B). The 2C T cells proliferated but failed to expand significantly in leukemia-bearing mice treated with vehicle control, as we have reported previously (Figures S3A and S3B) (Zhang et al., 2013). Interestingly, DMXAA treatment led to the accumulation of large numbers of fully divided 2C T cells in leukemia-bearing animals (Figures S3A and S3B). The frequency of 2C T cells isolated from AML-bearing mice that produced IFN-γ also was enhanced following DMXAA treatment (Figures S3C and S3D). As shown in Figure S3E, the amount of IFN-γ produced on a per-cell basis was also higher in 2C T cells from DMXAA- versus vehicle control-treated mice with AML. Together, these data demonstrate that activation of the STING pathway leads to an impressive expansion of functional leukemia-specific CD8+ T cells following adoptive transfer into mice with established AML.

To confirm that DMXAA-induced STING activation did not cause antigen-independent CD8+ T cell expansion in vivo, 2C and OT-1 T cells (the latter of which recognizes an irrelevant antigen derived from chicken ovalbumin) were co-transferred into mice. The following day, mice received C1498.SIY cells i.v. or remained leukemia-free. DMXAA or vehicle control was administered, and the frequencies of 2C and OT-1 T cells subsequently were analyzed. OT-1 T cells failed to expand in any group, regardless of AML cell inoculation or DMXAA administration (Figures S3F and S3G). This result conclusively demonstrates that STING activation results in activation of CD8+ T cells in an antigen-specific manner.

**STING Activation Enhances Survival of Leukemia-Bearing Mice**

To determine whether the powerful effect of STING activation on T cell priming correlated with improved disease control, animals with established C1498.SIY AML were treated with DMXAA or vehicle, and survival was assessed. As shown in Figure 3A, a single dose of DMXAA significantly prolonged survival of leukemia-bearing mice compared to those treated with vehicle control. In fact, ~60% of DMXAA-treated mice survived long term. Because SIY is an immunogenic model antigen, the ability of DMXAA to control the progression of parental C1498 AML also was tested. Here again, DMXAA-induced STING activation as a single therapeutic maneuver significantly prolonged survival, albeit to a lesser extent than in the C1498.SIY model (Figure 3B). Survival following DMXAA or vehicle control treatment was similarly poor in leukemia-bearing Tmem173+/− mice, demonstrating that the effect of DMXAA on disease control was STING dependent (Figures 3C and 3D).

To investigate the extent to which host type I IFN signaling was important for enhanced survival following STING activation, wild-type and Ifnar−/− mice were challenged with C1498 AML cells and treated with DMXAA or vehicle control. Survival was similar in vehicle control-treated wild-type and Ifnar−/− mice (Figure 3E), as previously shown (Figure 1B). As expected, DMXAA treatment led to a significant survival enhancement in wild-type mice, but survival also was extended to some degree in AML-bearing Ifnar−/− mice that received DMXAA (Figure 3E). Together, these data demonstrate that STING activation induces both type I IFN-dependent and -independent effects, which enhance leukemia-specific immunity and promote survival in AML-bearing mice.

Finally, because DMXAA is a selective agonist of murine, but not human, STING, the efficacy of a synthetic CDN STING agonist capable of activating both mouse and human STING was tested. This compound, dithio-(Rp, Rp)-[cyclic][A(2',5')pA(3',5')p] (ML RR-S2 CDA or simply CDA), has been shown
Figure 2. DMXAA-Induced STING Activation Potentiates Leukemia-Specific CD8+ T Cell Responses

C57BL/6 mice received 10⁶ C1498.SIY cells i.v. or remained leukemia-free. Mice were treated with 450 μg DMXAA or vehicle control i.v. (day 5), and cells were harvested for analysis one week later (day 12).

(A–C) Splenic or bone marrow SIY-specific CD8+ T cells were analyzed following SIY/Kb pentamer staining by flow cytometry. Representative plots show SIY/Kb pentamer staining (A). Numbers indicate frequency of SIY-specific CD8+ T cells. Frequency (left) and absolute number (right) of splenic SIY-specific CD8+ T cells are shown (B). Frequency of bone marrow SIY-specific CD8+ T cells is shown (C).

(D and E) Spleen cells from indicated mice were re-stimulated in vitro with SIY peptide, and the production of IFN-γ by CD8+ T cells was analyzed. Representative plots demonstrating IFN-γ production by CD8+ T cells are shown, and numbers indicate the frequency of IFN-γ+ CD8+ T cells (D). Frequency (left) and absolute number (right) of IFN-γ+ CD8+ T cells in the indicated groups are shown (E).

(B, C, and E) Data shown are pooled from at least two independent experiments, each with three mice per group, and represented as mean ± SEM (*p < 0.05 or **p < 0.01 for comparison of DMXAA- versus vehicle control-treated C1498.SIY-bearing mice).
to generate an anti-tumor T cell response and disease regression when administered intra-tumorally in solid tumor models (Corrales et al., 2015). To assess whether CDA treatment also would extend survival in animals with systemic AML, C57BL/6 mice were challenged with C1498.SIY cells i.v., followed by CDA or PBS on day 5. As shown in Figure 3F, CDA-treated leukemia-bearing mice survived significantly longer than controls.

DMXAA Therapy Requires Adaptive Immunity and Promotes Immunologic Memory against Native AML Antigens

To examine the role of the adaptive immune system in regulating the DMXAA effect on survival of AML-bearing mice, C57BL/6 and Rag2−/− mice (the latter of which lacks mature B and T cells) were challenged with C1498 or C1498.SIY cells, and they were treated with DMXAA or vehicle control. As demonstrated previously, DMXAA treatment enhanced survival of C57BL/6 mice following a systemic inoculation of C1498.SIY (Figure 4A) or parental C1498 cells (Figure 4B), when compared to vehicle control-treated animals. However, the survival of leukemia-bearing Rag2−/− mice was identical in DMXAA- and control-treated mice (Figures 4A and 4B), indicating that the therapeutic effect of STING activation in AML-bearing animals requires adaptive immunity.

It was next determined whether functional memory was generated against native C1498-expressed antigens following DMXAA treatment. C57BL/6 mice that survived a primary i.v. C1498.SIY challenge after treatment with DMXAA received a subsequent challenge with parental C1498 cells 100 days later. A second group of C57BL/6 mice received a primary i.v. C1498 cell challenge simultaneously as a comparator cohort. DMXAA treatment of leukemia-bearing mice promoted a remarkable survival benefit following AML cell re-challenge, clearly demonstrating that effective memory responses are generated against native C1498 antigens following STING activation (Figure 4C).

STING Activation Is Effective in a Genetically Engineered AML Model

To assess whether STING activation would be effective in a second AML model, the Cbfβ-MYH11/Mpl-induced mouse leukemia model (CMM+) was utilized. This genetically engineered...
AML model mimics human inv(16) AML. Mice with established CMM+ leukemia received DMXAA or vehicle control treatment weekly. STING activation resulted in a significant decrease in the frequency of AML cells in spleens of treated mice (Figures 5A and 5B), with a corresponding decrease in splenomegaly (data not shown). These anti-tumor effects also translated into extended survival of DMXAA-treated CMM+ mice compared to controls (Figure 5C) and demonstrate that the effectiveness of immunotherapy with STING agonists is not limited to a single AML model.

**DISCUSSION**

The observation that AML failed to induce a host type I IFN response is indicative of an impaired capacity of the innate immune system to sense a disseminated leukemia, possibly through lack of STING activation. The ability of leukemia cells inoculated s.c., but not i.v., to induce type I IFN expression (Figure 1A), as well as the finding that systemic AML progressed independently of type I IFN signaling on host cells (Figure 1B), argues that the disseminated nature of leukemia may be the critical factor. Precisely why systemic leukemia fails to activate type I IFN is not known and is being actively explored in our laboratory. Regardless, systemic administration of a STING agonist stimulated expression of type I IFN and other cytokines that may have contributed to its effectiveness. DMXAA demonstrated impressive therapeutic efficacy in two aggressive syngeneic AML models, potently activated leukemia antigen-specific T cells, and induced immunological memory against naturally expressed leukemia antigens.

The therapeutic effect of DMXAA was STING dependent and partially required type I IFN responsiveness in host cells. The latter finding is interesting, and it suggests that additional cytokines, such as TNF-α, may be functioning downstream of STING activation to promote anti-leukemia immunity. In fact, DMXAA initially was shown to mediate cancer regression through an effect on tumor vasculature, largely through TNF-α (Joseph et al., 1999; Zhao et al., 2002). The extent to which TNF-α and other cytokines are involved in the DMXAA effect in leukemia-bearing mice is currently being explored. That STING activation stimulates production of a variety of cytokines aside from type I IFN argues that this approach might be superior to treatment with type I IFN alone, which has demonstrated limited clinical efficacy in AML (Anguille et al., 2011; Smits et al., 2013). Furthermore, STING activation is more effective in controlling established leukemia than approaches targeting other nucleic acid-sensing receptors, including Toll-like receptor 3 (TLR3) (data not shown) and TLR9 (Hossain et al., 2014). Another important observation was the requirement for adaptive immunity following STING activation, as DMXAA treatment was completely ineffective when administered to leukemia-bearing Rag2−/− mice. This contrasts what has been reported in some solid tumor models, in which a partial T cell-independent effect of STING agonists has been described (Corrales et al., 2015). The T cell-independent effect of STING activation in solid tumors may be related to the well-known anti-angiogenic properties of IFN-β and vascular destructive effects of TNF-α (Spaapen et al., 2014; Zhao et al., 2002), which are perhaps more relevant in neo-vascularized solid cancers compared to acute leukemia.

Figure 4. DMXAA Therapy Requires Adaptive Immunity and Generates Effective Memory Responses to Naturally Expressed AML Antigens

(A and B) C57BL/6 or Rag2−/− mice received 10⁶ C1498.SIY (A) or C1498 (B) cells i.v. On day 5 (A) or on days 3 and 10 (B) mice were treated with DMXAA or vehicle control i.v., and survival was assessed (***p < 0.001 for survival in DMXAA- versus vehicle-treated C57BL/6 mice).

(C) DMXAA-treated survivors of a primary C1498.SIY cell challenge received 10⁶ parental C1498 cells i.v. approximately 100 days following the initial C1498.SIY cell inoculation. Naive C57BL/6 mice inoculated with C1498 cells served as controls (****p < 0.0001 for survival in long-term C1498.SIY survivors versus leukemia-naive mice following inoculation with C1498 cells).
Several groups have reported recently that STING agonists, administered directly into the tumor as single agents, or s.c. with irradiated tumor cells, are capable of inducing potent immunity against a variety of transplanted solid cancers (Corrales et al., 2015; Fu et al., 2015). Locally administered STING agonists also augment anti-tumor immune responses following radiation therapy (Deng et al., 2014). Our recent work has indicated that a unique immune evasion mechanism is active in AML in which antigen-specific CD8+ T cells undergo abortive proliferation and are rapidly deleted from leukemia-bearing animals (Zhang et al., 2013). The ability of STING agonists to overcome this dense T cell-tolerant state that exists in AML-bearing mice is remarkable.

A potential limitation of this study is the use of transplantable C1498 and CMM+ leukemia models, where disease induction through i.v. inoculation likely fails to recapitulate several biological aspects of AML. Investigating the role of STING pathway activation in more physiologically relevant AML models will be an important area of future study. Furthermore, given the broad efficacy of localized STING therapy across solid tumor models, we speculate that systemic STING activation will be efficacious in autochthonous AML models and also against other disseminated hematological malignancies.

Modified CDNs have been developed that bind both murine and all human STING alleles, and, like DMXAA, they potently induce activation of the STING axis. CDA, a lead CDN, has shown potent anti-tumor activity in several studies (Corrales et al., 2015; Fu et al., 2015). Systemic delivery of CDA to AML-bearing mice in our study also was effective at improving survival of AML-bearing animals, similar to DMXAA treatment. The maximally effective dose and schedule of the CDA compound, when administered systemically, will require further study in order to define its optimal effect in vivo.

Although STING activation appears to be broadly effective as cancer immunotherapy, counter-regulatory immune evasion pathways, including IFN-γ-induced PD-L1 upregulation in the tumor environment (Spranger et al., 2013; Fu et al., 2015), enhanced production of the immunosuppressive indolamine-2,3-dioxygenase (IDO) enzyme (Spranger et al., 2013), and
the influx of regulatory T cells into the tumor environment (Spranger et al., 2013) may limit its use as a single agent. Thus, defining immune escape pathways that are activated following STING agonist therapy and developing therapeutic combination strategies to overcome them will be important to consider in future studies.

As the development of STING agonists for use in humans evolves, defining the efficacy and underlying mechanisms of action in both solid and hematologic cancers will be crucial. The focus of STING pathway agonists for clinical use has thus far centered on their role as vaccine adjuvants and as cancer immunotherapeutic agents for treatment of solid tumors. However, our results demonstrate similar impressive improvements in survival and immune responses in pre-clinical AML models, and they provide strong rationale for the clinical translation of STING agonists as immune therapy for leukemia and possibly other hematologic malignancies.

**EXPERIMENTAL PROCEDURES**

**Mice and Leukemia Cell Lines**

C57BL/6 (H-2b) mice, aged 6–12 weeks, were purchased from Taconic. Tmem173<sup>−/−</sup> and Ifnar<sup>−/−</sup> mice have been reported previously (Shikawa and Barber, 2008; Müller et al., 1994), and they were provided by Y.-X. Fu (University of Chicago). IL-12 yellow fluorescent protein (YFP) reporter mice were purchased from Jackson ImmunoResearch Laboratories. Ragg<sup>−/−</sup> and 2C/C57BL/6 mice were bred in our facility. All mice were maintained in a specific pathogen-free environment. The C1498 cell line of C57BL/6 origin (H-2b<sup>−</sup>) was generated in our laboratory as previously described (Zhang et al., 2009).

**Real-Time qPCR Analysis**

C57BL/6, Tmem173<sup>−/−</sup>, or Ifnar<sup>−/−</sup> mice were treated with DMXAA or vehicle control, and spleen cells were harvested 6 hr later. For the measurement of cytokine expression in leukemia-bearing mice, 5 × 10<sup>6</sup> C1498 cells were injected by i.v. or s.c. route. Spleen or lymph node cells, respectively, were harvested 72 hr later. All samples were re-suspended in Trizol (Life Technologies) and total RNA was isolated via chloroform extraction. The cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR reactions were performed using TaqMan Gene Expression Master Mix (A&B), and a 7300 Real Time PCR system (A&B) was then performed for Ifnb, Trif, and Il6.

**ELISA**

C57BL/6 mice were treated with DMXAA or vehicle control and serum was obtained 6 hr later. ELISA was performed for IFN-β, TNF-α, and IL-6 using pre-coated plates (BioLegend) according to the manufacturer’s protocol.

**AML Models**

C1498 or C1498.SIY cells (10<sup>6</sup>) were injected i.v. into the lateral tail vein of recipient mice, and survival or immune responses were analyzed. To induce localized C1498 tumors, 10<sup>6</sup> C1498 cells were inoculated s.c. in the lower lateral abdominal wall. Tumor volume was measured two to three times weekly, and animals were euthanized when the maximal tumor diameter reached 20 mm or when tumor ulceration or other clinical signs of distress developed. To generate the Cbta-MYH11/Mpl-induced mouse leukemia model (CMM*), polyinosinic-polycytidylic acid (poly(I:C)) was administrated to Cbta<sup>−/−</sup>Myh11<sup>−/−</sup>/Mpl<sup>−/−</sup> mice to induce expression of core-binding factor β-smooth muscle myosin heavy chain (Kuo et al., 2006). Then 2 weeks later, bone marrow cells were harvested and transduced with the retroviral MIG-Mpl vector and GFP genes to generate a transplantable Cbta-MYH11/Mpl<sup>−/−</sup> mouse AML, as previously described (Hossain et al., 2014).

**In Vivo Administration of STING Agonists**

DMXAA (Sigma-Aldrich) was dissolved in sterile 7.5% NaHCO<sub>3</sub> and 450 µg was injected i.v. on the day(s) indicated in each experiment. CDA was provided by Aduro Biotech and diluted in sterile PBS; 100 µg was injected i.v.

**Pentamer Staining and Flow Cytometric Analysis**

SIY<sup>R</sup>κ<sup>P</sup> pentamers were purchased from Proimmune. Spleen or bone marrow cells from experimental animals were stained with SIY<sup>R</sup>κ<sup>P</sup>-PE pentamers according to the manufacturer’s protocol, followed by anti-CD4-PerCP-Cy5.5 and anti-B220-PerCP-Cy5.5 antibodies (to exclude CD4<sup>+</sup> T cells and B cells), anti-CD8-FITC, and anti-Thy1.2-APC antibodies. Non-viable cells were excluded from the analysis with a viability dye (Life Technologies). Flow cytometry was performed on a Fortessa cytometer (BD Biosciences) with BD FACSDiva software. Data analysis was performed with FlowJo software (Tree Star) to identify the frequencies and absolute numbers of SIY-specific CD8<sup>+</sup> T cells.

**Adaptive Transfer of 2C T Cells into Leukemia-Bearing Mice**

The 2C CD8<sup>+</sup> TCR Tg T cells, specific for the SIY antigen in the context of H<sup>2</sup>B, were purified from spleens of 2C/C57BL/6 mice by positive selection using a CD8 microbead kit, according to the manufacturer’s protocol (Milteny). Purified 2C T cells were labeled with CellTrace Violet (CTV) (Life Technologies) according to the manufacturer’s protocol. CTV-labeled 2C T cells (10<sup>5</sup>) were injected into C57BL/6 mice i.v.; 1 day later, mice received 10<sup>5</sup> C1498.SIY cells i.v. DMXAA or vehicle was administered as indicated. Then 7 days later, spleens were harvested and stained with anti-CD8, anti-Thy1.2, and anti-1B2 antibodies (the 1B2 monoclonal antibody recognizes the 2C TCR), followed by secondary labeling with streptavidin-PE. Flow cytometry analysis was then performed.

**Adaptive Co-transfer of OT-1 and 2C T Cells**

OT-1 and 2C T cells were isolated from OT-1 (Thy1.1<sup>+</sup>) and 2C (Thy1.2<sup>+</sup>) mice using a CD8 microbead kit (Milteny). Purified OT-1 and 2C T cells were mixed at a 1:1 ratio, and 2 × 10<sup>7</sup> T cells (10<sup>5</sup> 2C and 10<sup>5</sup> OT-1 T cells) were co-transferred into groups of C57BL/6 mice. Then 24 hr later, half of the mice received C1498.SIY cells i.v., while the other half remained leukemia free. DMXAA or vehicle was administered on day 5, and, 7 days later, spleen cells from each group of mice were analyzed by flow cytometry after cell surface staining with anti-CD8, anti-Thy1.2, anti-Thy1.1, and anti-1B2 antibodies in order to identify the frequencies of OT-1 and 2C T cells present.

**Intracellular Cytokine Staining**

Spleens were harvested from leukemia-bearing mice 5–7 days following the administration of DMXAA or vehicle. Spleen cells (~10<sup>9</sup>) from individual mice were plated in flat-bottomed 48-well tissue culture plates and stimulated with medium supplemented with SIY peptide (500 nM) at 37°C for 1 hr, followed by 4 hr in the presence of 1 µg/ml GolgiPlug (BD Biosciences). Cells were re-collected and stained with anti-CD8 and anti-1B2 antibodies (for 2C transfer experiments) or anti-CD8 and anti-Thy1.2 antibodies (for analysis of endogenous SIY-specific CD8<sup>+</sup> T cell responses). After washing, cells were fixed and permeabilized for 30 min at 4°C (eBiosciences FoxP3 intracellular staining kit) and stained overnight with anti-IFN-γ-PE antibody. After washing, cells were analyzed for IFN-γ production by flow cytometry after gating on 2C T cells (Thy1.2/CD8<sup>+</sup>1B2<sup>+</sup> cells) or endogenous CD8<sup>+</sup> T cells (Thy1.2/CD8<sup>+</sup>6<sup>+</sup> cells).

**Statistics**

Student’s t tests, ANOVA with Bonferroni correction, Mann-Whitney, and Kruskal-Wallis test were used for statistical analysis. For survival analysis, Kaplan-Meier curves were generated and log-rank test was used to compare survival between groups. Statistics were performed using Graph Prism software. A p value of <0.05 was considered statistically significant.

**Study Approval**

Animal experimentation was carried out under a protocol approved by an Institutional Animal Care and Use Committee.
SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.023.

AUTHOR CONTRIBUTIONS

E.C. designed and executed experiments, analyzed data, and drafted the manuscript. X.C. designed and executed experiments and reviewed the manuscript. D.E.K., M.K., and P.D. designed experiments and reviewed the manuscript. J.K. designed experiments, analyzed data, and drafted and reviewed the manuscript.

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