

IMMUNOTHERAPY

NK cells mediate clearance of CD8⁺ T cell-resistant tumors in response to STING agonistsChristopher J. Nicolai¹, Natalie Wolf¹, I-Chang Chang¹, Georgia Kirn¹, Assaf Marcus¹, Chudi O. Ndubaku², Sarah M. McWhirter², David H. Raulet^{1*}Copyright © 2020
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Several immunotherapy approaches that mobilize CD8⁺ T cell responses stimulate tumor rejection, and some, such as checkpoint blockade, have been approved for several cancer indications and show impressive increases in patient survival. However, tumors may evade CD8⁺ T cell recognition via loss of MHC molecules or because they contain few or no neoantigens. Therefore, approaches are needed to combat CD8⁺ T cell-resistant cancers. STING-activating cyclic dinucleotides (CDNs) are a new class of immune-stimulating agents that elicit impressive CD8⁺ T cell-mediated tumor rejection in preclinical tumor models and are now being tested in clinical trials. Here, we demonstrate powerful CDN-induced, natural killer (NK) cell-mediated tumor rejection in numerous tumor models, independent of CD8⁺ T cells. CDNs enhanced NK cell activation, cytotoxicity, and antitumor effects in part by inducing type I interferon (IFN). IFN acted in part directly on NK cells in vivo and in part indirectly via the induction of IL-15 and IL-15 receptors, which were important for CDN-induced NK activation and tumor control. After in vivo administration of CDNs, dendritic cells (DCs) up-regulated IL-15R α in an IFN-dependent manner. Mice lacking the type I IFN receptor specifically on DCs had reduced NK cell activation and tumor control. Therapeutics that activate NK cells, such as CDNs, checkpoint inhibitors, NK cell engagers, and cytokines, may represent next-generation approaches to cancer immunotherapy.

INTRODUCTION

Recent breakthroughs in tumor immunology have provided novel immune-based therapeutics, extending patient lives and, in some cases, resulting in what appear to be permanent remissions (1, 2). Most immunotherapy protocols aim to augment CD8⁺ T cell responses by targeting immune inhibitory pathways, leading to greater T cell activation and tumor destruction (3, 4). However, tumors may evade the CD8⁺ T cell response via selective or complete loss of major histocompatibility complex (MHC) class I expression (5–7) or because they express few or no neoantigens (8) and may consequently be refractory to CD8⁺ T cell-dependent therapies. Therefore, knowledge of how the immune system can be mobilized to kill CD8⁺ T cell-resistant tumors is needed to address these potential escape mechanisms and design next-generation immunotherapies.

Natural killer (NK) cells are cytotoxic innate lymphocytes that are important for killing virus-infected cells and tumor cells (9–11). Unlike T cells, which target unique peptide antigens displayed on MHC molecules, NK cells recognize abnormally expressed, stress-induced ligands on unhealthy cells (11–14) and/or cells that have lost MHC class I (15–18). Furthermore, NK cells produce cytokines and chemokines that enhance recruitment and maturation of dendritic cells (DCs) (19, 20), promoting adaptive immune responses. These features enable NK cells to increase adaptive immune responses to tumors and directly kill tumors that have escaped T cell responses, making NK cells exciting targets for immunotherapy.

The cyclic guanosine monophosphate-adenosine monophosphate synthase–stimulator of interferon genes (cGAS-STING) pathway is an innate immune sensing pathway that senses cytosolic DNA, resulting in production of type I interferon (IFN) and proinflammatory

cytokines and chemokines (21, 22). Upon binding double-stranded DNA, the cGAS enzyme generates the second messenger 2', 3'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) (21, 23, 24). cGAMP binds and activates the endoplasmic reticulum membrane protein STING (21, 22), triggering recruitment of TANK-binding kinase 1 (TBK1) and phosphorylation and activation of interferon regulatory factor 3 and nuclear factor κ B transcription factors (21).

The cGAS-STING pathway is essential for sensing certain viral and bacterial pathogens (21) but is also activated in tumor cells (25). Moreover, mice lacking functional STING are more susceptible to both transplanted (26, 27) and carcinogen-induced tumors (28). Cytosolic tumor DNA is thought to initiate the response (26, 27) and induces production of cGAMP, which is transferred to other cells to activate STING (27, 29, 30), promoting cytokine production and activation of antitumor responses by both CD8⁺ T cells (26, 31) and NK cells (27). However, the amounts of cGAMP made or transferred appear to be limiting for inducing a maximally potent antitumor response. Injection of cGAMP or other STING agonists directly into tumors induces a powerful antitumor response leading to tumor rejection in various tumor transplant models of cancer (32–36). On the basis of these findings, STING agonists are currently being tested in clinical trials.

The antitumor effects of STING agonists have primarily been attributed to CD8⁺ T cells (32, 35, 37), whereas their impact on other cells, such as NK cells, remains poorly defined. STING activation potentially induces multiple inflammatory mediators, including type I IFNs (22), which play central roles in NK cell biology, including maturation, homeostasis, and activation (38). In this study, we have investigated the role of NK cells in mediating tumor rejection after cyclic dinucleotide (CDN) therapy, independent of the CD8⁺ T cell response. Our results demonstrate powerful CD8-independent antitumor responses mediated by NK cells that are induced by therapeutic applications of CDNs in numerous cancer models, including both MHC I-deficient and MHC I-sufficient tumor models.

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RESULTS**Successful immunotherapy of MHC class I-deficient tumors by CDNs occurs independently of CD8⁺ T cells**

To examine the CD8⁺ T cell-independent antitumor effects of intratumoral CDN injection, we used CRISPR-Cas9 to disrupt *B2m* in multiple tumor cell lines, generating cells with severely diminished levels of cell surface MHC I molecules (Fig. 1A and fig. S1). Such tumor models have potential clinical relevance in light of evidence that MHC I deficiency is selected for when T cell responses against tumors are induced and is common in certain cancers (5–7, 39–41). Tumors were established with a high dose of MHC I-deficient cells injected subcutaneously in syngeneic mice, and treated intratumorally once, or in some cases three times, with mixed-linkage (2'3') RR cyclic diadenosine monophosphate (c-di-AMP) (33) (also known as ADU-S100 and hereafter referred to as “CDN”) or phosphate-buffered saline (PBS). The dose of CDN used has been shown to be optimal for CD8⁺ T cell responses (32). CDN injections resulted in regression and severely delayed tumor growth in each of six *B2m*^{-/-} tumor models tested, representing multiple types of cancer (Fig. 1B). In all but one model, there was a substantial incidence of long-term remissions as a result of single-agent intratumoral administration of CDN, with no evidence of renewed tumor growth for the remainder of the study (50 to 100 days). The impact of CDNs was abrogated in *Sting*^{gt/gt} mice in both tumor models subsequently tested, demonstrating the role of host STING in the responses (Fig. 1C). Depletion of CD8⁺ T cells using CD8b.2 antibody (fig. S2) did not diminish tumor rejection in either of the two models tested, consistent with the absence of MHC I molecules on the tumor cells (Fig. 1D). These data showed that intratumoral injections of CDNs trigger potent antitumor effects independently of CD8⁺ T cells.

CDN-induced rejection of MHC I-deficient tumors depends on NK cells

To test the role of NK cells, we used NK1.1 antibody to deplete mice of NK cells before tumor implantation and subsequent CDN treatment. NK depletion (fig. S2) resulted in rapid tumor growth in all five tumor models tested, including MC-38-*B2m*^{-/-} (colorectal), B16-F10-*B2m*^{-/-} (melanoma), CT26-*B2m*^{-/-} (colorectal), C1498-*B2m*^{-/-} (leukemic), and RMA-*B2m*^{-/-} (lymphoma) tumor models (Fig. 2A). For the RMA-*B2m*^{-/-} lymphoma line, CDN therapy was also defective in NK-diphtheria toxin alpha (DTA) mice, which specifically lack NK cells because of DTA expression only in NKp46⁺ cells (Fig. 2B) (42). CDN-induced tumor rejection also occurred in *Rag2*^{-/-} mice, which lack T and B cells but was strongly diminished in NK-depleted *Rag2*^{-/-} mice (Fig. 2C), or in *Rag2*^{-/-}*Il2rg*^{-/-} mice, which lack NK cells and other innate lymphoid cells in addition to lacking T and B cells (Fig. 2D). Thus, CDNs mobilize powerful NK responses against MHC I-deficient tumors that are quite effective in the absence of T and B cells.

Without NK cells, T cells, or B cells, as in *Rag2*^{-/-}*Il2rg*^{-/-} mice, CDN injections caused a residual delay in tumor growth (Fig. 2, C and D, and fig. S1B). Consistent with previous evidence that STING agonists induce an immediate local hemorrhagic necrosis in tumors, mediated by tumor necrosis factor- α (TNF- α) (34), the CDN-induced delay in the growth of RMA-*B2m*^{-/-} tumors was eliminated when TNF- α was neutralized in *Rag2*^{-/-}*Il2rg*^{-/-} mice (fig. S1B). The delay in tumor growth in *Rag2*^{-/-}*Il2rg*^{-/-} mice was transient, and none of the mice survived, showing that robust antitumor effects depended on lymphocytes.

Many tumor cells express high MHC I but are nevertheless sensitive to NK cells due to high expression of NK-activating ligands (18, 43). An important question was whether CDN-induced, NK-mediated, antitumor effects would be effective against MHC I-high tumor cells that are NK sensitive. To address this question, we used the wild-type (WT) (*B2m*^{+/+}) MC-38 line, which is MHC I high (Fig. 1A) but which NK cells kill effectively in vitro because, at least in part, of the expression of natural-killer group 2, member D (NKG2D) ligands by these tumor cells (43). In *Rag2*^{-/-} mice, which lack all T and B cells, CDN treatment was effective in delaying growth of MC-38 tumors and even resulted in a few long-term survivors (Fig. 2E). NK depletion resulted in rapid tumor growth and eliminated any long-term survivors. Thus, NK cells can reject MHC I⁺ MC-38 tumor cells after CDN injections, even in the complete absence of T cells. We conclude that CDN-induced NK responses are effective not only against MHC I-deficient tumors but also against tumors that are NK sensitive because, for example, of expression of NK-activating ligands.

NK cells are activated by intratumoral CDN injections and accumulate within tumors

To address the impact of CDN treatments on NK cells, we examined markers of NK cell activation among tumor-infiltrating, draining lymph node, and splenic NK cells 1 day after treatment, with no additional stimulation ex vivo. Compared with NK cells within PBS-treated tumors, NK cells within CDN-treated tumors had increased levels of IFN- γ , the degranulation marker CD107a, granzyme B, and Sca-1 (Fig. 3A and fig. S3), demonstrating an increased degree of NK cell activation. Furthermore, NK cells accumulated among CD45⁺ cells within CDN-treated tumors (Fig. 3B). The relative increase of NK cells within tumors coincided with an increase in Ki67 expression (Fig. 3B and fig. S4), suggesting that CDNs promote NK cell proliferation in addition to activation.

CDN treatment also caused NK cell activation in the tumor-draining lymph node and even in the spleen (Fig. 3A), suggesting that intratumoral injection of CDNs resulted in systemic NK activation. Consistent with systemic activation, we found that splenocytes harvested from tumor-bearing CDN-treated mice, but not PBS-treated control mice, exhibited detectable cytotoxicity against RMA-*B2m*^{-/-} tumor cells ex vivo (Fig. 3C). Depleting NK cells after harvest abolished the killing.

On the basis of these observations, we tested whether systemic NK cell activation induced by CDN administered locally in one tumor would also trigger antitumor responses in an untreated distal tumor. We established C1498-*B2m*^{-/-} tumors on both flanks of *Rag2*^{-/-} mice and treated one tumor with PBS or CDN. As expected, on the basis of the results in Fig. 1B, intratumoral CDN treatment caused substantial tumor regression in the injected tumor (Fig. 3D). There was also a substantial growth delay in the untreated distal (contralateral) tumor compared with PBS, showing that intratumoral CDN treatments induce systemic antitumor effects, independent of T and B cells. Similar results were obtained with a separate tumor model, B16-F10-*B2m*^{-/-}, in T cell-depleted WT mice (fig. S5). When the *Rag2*^{-/-} mice with C1498-*B2m*^{-/-} tumors were depleted of NK cells, the antitumor effects at both the treated and distal tumor were severely abrogated. Because these mice lack all T and B cells, the results demonstrate that the systemic, CDN-induced effects were mediated by NK cells independently of T cells (Fig. 3D). In conclusion, intratumoral CDN treatment induced NK activation within tumors and, to some extent, systemically, enhanced ex vivo NK killing capacity, and exerted antitumor effects on a distant tumor.

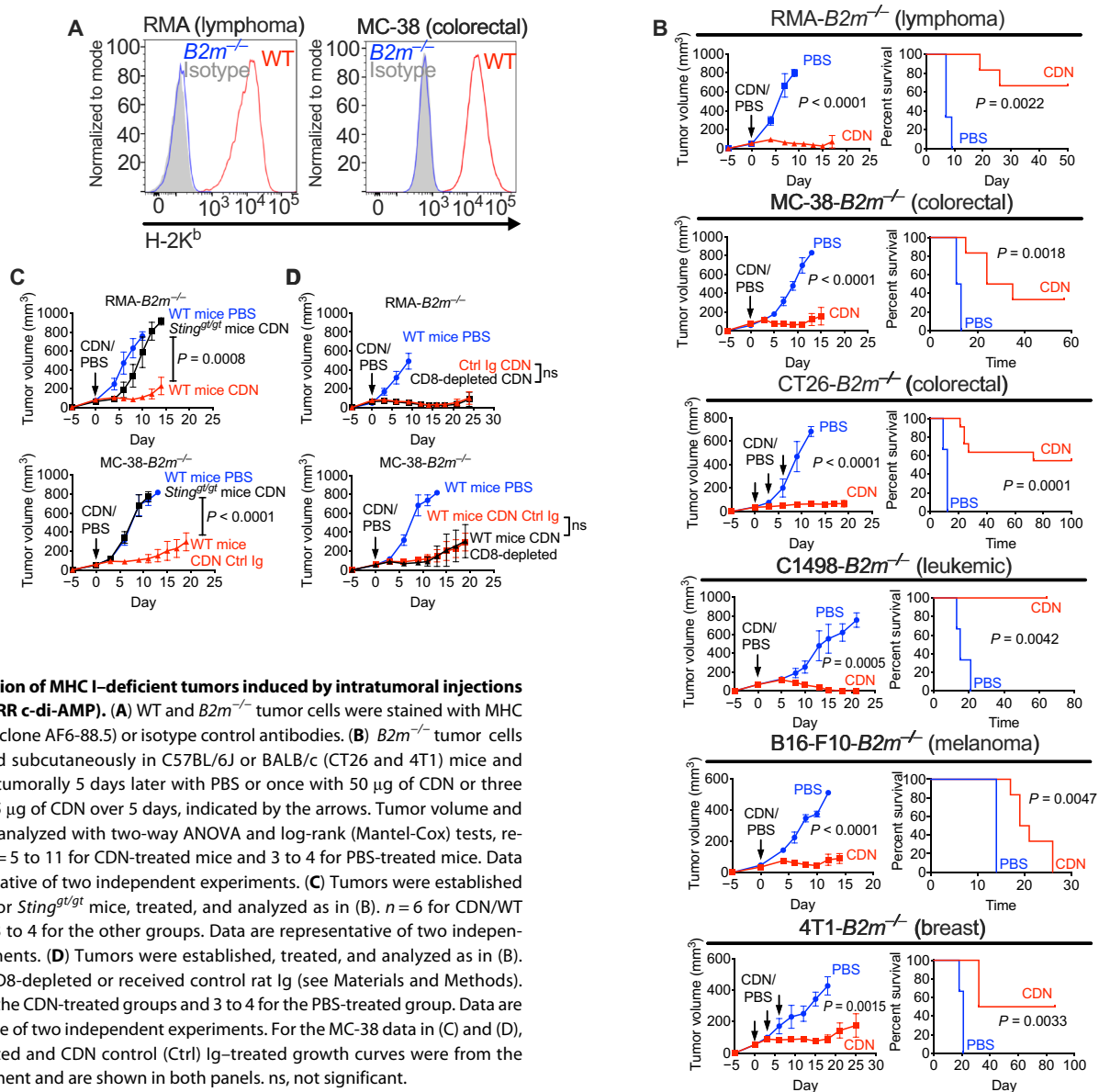


Fig. 1. Rejection of MHC I-deficient tumors induced by intratumoral injections of CDN (2'3' RR c-di-AMP). (A) WT and $B2m^{-/-}$ tumor cells were stained with MHC class I (H-2K^b clone AF6-88.5) or isotype control antibodies. (B) $B2m^{-/-}$ tumor cells were injected subcutaneously in C57BL/6J or BALB/c (CT26 and 4T1) mice and treated intratumorally 5 days later with PBS or once with 50 μ g of CDN or three times with 25 μ g of CDN over 5 days, indicated by the arrows. Tumor volume and survival was analyzed with two-way ANOVA and log-rank (Mantel-Cox) tests, respectively. $n = 5$ to 11 for CDN-treated mice and 3 to 4 for PBS-treated mice. Data are representative of two independent experiments. (C) Tumors were established in C57BL/6J or *Sting*^{90/9t} mice, treated, and analyzed as in (B). $n = 6$ for CDN/WT groups and 3 to 4 for the other groups. Data are representative of two independent experiments. (D) Tumors were established, treated, and analyzed as in (B). Mice were CD8-depleted or received control rat Ig (see Materials and Methods). $n = 5$ to 8 for the CDN-treated groups and 3 to 4 for the PBS-treated group. Data are representative of two independent experiments. For the MC-38 data in (C) and (D), the PBS-treated and CDN control (Ctrl) Ig-treated growth curves were from the same experiment and are shown in both panels. ns, not significant.

NK cell activation and tumor rejection are dependent on type I IFN acting on host cells

Consistent with the known role of STING activation in type I IFN production (22), we observed a marked increase in *Ifnb1* transcripts within tumors 24 hours after CDN treatment compared with PBS-treated controls (Fig. 4A). Serum of CDN-treated mice also contained high levels of IFN- β shortly after treatment (Fig. 4B). These data are consistent with a recent study showing low but detectable circulating IFN- β in patients treated with CDNs combined with anti-programmed death-1 (PD-1) (44). The systemic antitumor effects reported in Fig. 3 may be explained, at least in part, by the induction of significant levels of systemic IFN- β by local CDN treatments. NK cell activation was strongly dependent on type I IFN because CDN-treated *Ifnar1*^{-/-} mice, which lack functional type I IFN receptor, did not display increases in IFN- γ , CD107a, granzyme B, or Sca-1 in response to CDNs compared with WT controls (Fig. 4C and fig. S6). Similar results were obtained in the tumor-draining lymph nodes and spleens of WT

mice injected with interferon-alpha/beta receptor 1 (IFNAR1)-blocking antibodies (fig. S7, A and B). Furthermore, splenocytes from CDN-treated *Ifnar1*^{-/-} mice, or WT mice given IFNAR1-blocking antibodies, were unable to kill RMA- $B2m^{-/-}$ tumor cells ex vivo, unlike splenocytes from CDN-treated WT mice (Fig. 4D and fig. S7C). Therefore, type I IFN action is essential for NK cell activation and deployment of effector functions after CDN injections.

In terms of tumor rejection, both MHC I-deficient tumor cell lines tested, RMA- $B2m^{-/-}$ and MC-38- $B2m^{-/-}$, were refractory to CDN therapy in *Ifnar1*^{-/-} mice (Fig. 4E). Knocking out *Ifnar1* in RMA- $B2m^{-/-}$ tumor cells had no effect on tumor rejection (fig. S7D), indicating that type I IFN action on host cells, rather than tumor cells, is necessary for the response. IFNAR1 neutralization also abrogated the antitumor effects of CDN therapy for RMA- $B2m^{-/-}$ tumors (fig. S7E), suggesting that acute effects of CDN-induced type I IFN, rather than developmental or homeostatic effects, are key to the antitumor response. NK depletion combined with IFNAR1

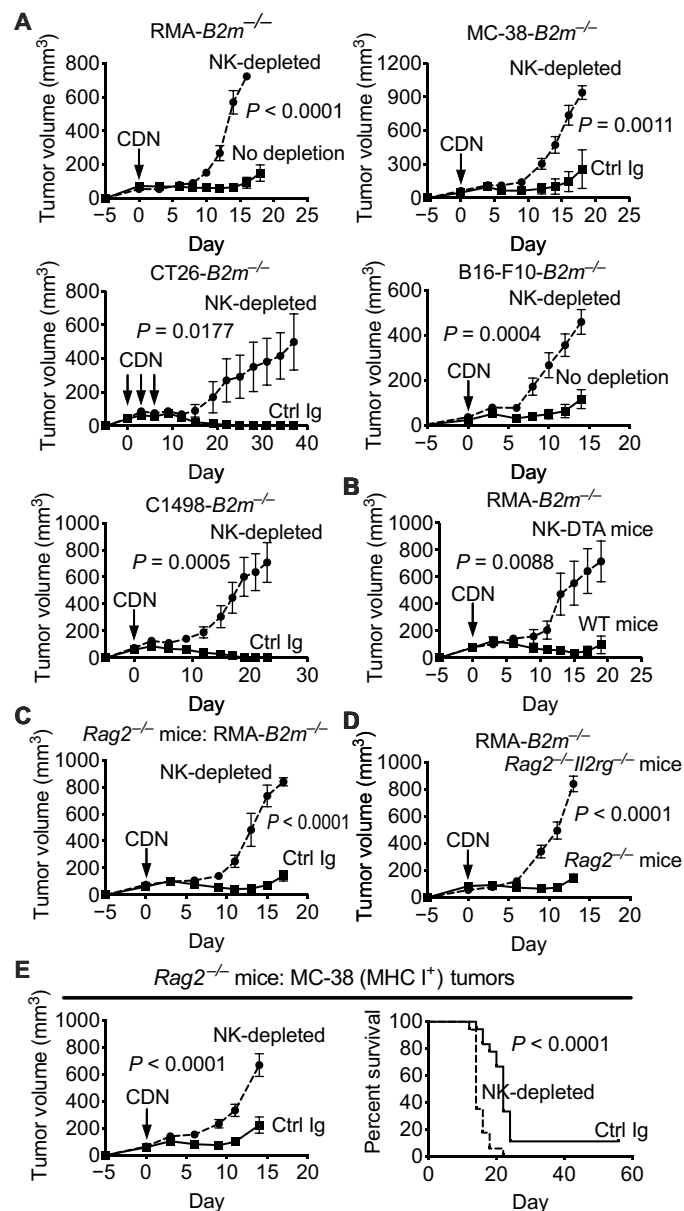


Fig. 2. NK dependence of tumor rejection induced by CDNs. (A) C57BL/6J or BALB/c (CT26), (B) NK-DTA, (C and D) *Rag2*^{-/-}, or (D) *Rag2*^{-/-}*Il2rg*^{-/-} mice were injected subcutaneously with tumor cells of the types indicated, and tumors were allowed to establish for 5 days. In some experiments (A, C, and E), mice were NK-depleted (see Materials and Methods). Tumors were treated and analyzed as described in Fig. 1B. (E) *B2m*^{+/-} MC-38 tumors (MHC I⁺) were established in *Rag2*^{-/-} mice that were NK-depleted or not, CDN-treated, and analyzed as in Fig. 1B. For (A) to (D), data are representative of two to three independent experiments. *n* = 5 to 9. For (E), data were combined from three independent experiments. *n* = 18.

blockade had no greater effect than either treatment alone in *Rag2*^{-/-} mice (Fig. 4F), supporting the conclusion that the NK-mediated antitumor activity is strongly dependent on type I IFN.

Type I IFN acts directly on NK cells to mediate the antitumor response

We initially used bone marrow chimeras between WT and *Ifnar1*^{-/-} mice to address the cell types on which type I IFN acts to mediate

NK-dependent antitumor responses. The chimeric mice, which showed near-complete chimerism (fig. S8), were implanted with RMA-*B2m*^{-/-} tumor cells, and the established tumors were subjected to intratumoral CDN therapy. Tumor rejection in *Ifnar1*^{-/-} → *Ifnar1*^{-/-} and *Ifnar1*^{-/-} → WT chimeras was largely impaired compared with control chimeras, whereas WT → *Ifnar1*^{-/-} chimeras behaved like WT → WT controls. These data argue that the action of type I IFN on hematopoietic cells is necessary and mostly sufficient for tumor rejection (Fig. 4G). In this and another experiment, there were hints that type I IFN acting on radioresistant cells may play a minor role in the rejection response, such as the slight delay in tumor growth in *Ifnar1*^{-/-} → WT chimeras compared with *Ifnar1*^{-/-} → *Ifnar1*^{-/-} chimeras in Fig. 4G (*P* = 0.036).

To examine whether direct effects of type I IFN on NK cells were important for CDN-induced antitumor effects, we used *Ncr1-iCre*, *Ifnar1*^{fl/fl} mice, in which *Ifnar1* expression is defective only in NK cells (fig. S9). *Ifnar1* deletion in NK cells was highly efficient and specific as it was not evident in T cells (fig. S9). In vivo, *Ncr1-iCre*, *Ifnar1*^{fl/fl} mice were unable to control tumor growth after CDN therapy and had reduced overall survival, indicating the importance of direct type I IFN action on NK cells for tumor rejection (Fig. 5A). However, the defect in tumor control was not as substantial as in NK-depleted mice (Fig. 5A) or as in *Ifnar1*^{-/-} mice (Fig. 5B), suggesting that type I IFN boosts NK-mediated tumor rejection in part by acting indirectly on non-NK cells. Furthermore, NK cells in the tumor-draining lymph nodes of CDN-treated *Ncr1-iCre*, *Ifnar1*^{fl/fl} mice had decreased levels of IFN-γ, CD107a, granzyme B, and Sca-1 (Fig. 5C and fig. S10), although they were not reduced to the control levels observed in *Ifnar1*^{fl/fl} mice (no Cre) treated with PBS. We also observed that *Ifnar1* deletion specifically in NK cells resulted in a sharp reduction in CDN-induced cytotoxicity of splenocytes against RMA-*B2m*^{-/-} tumor cells, although a very small amount of cytotoxicity may remain (Fig. 5B). These data show that type I IFN acts directly on NK cells but likely also acts on another cell type(s) to indirectly enhance NK cell activation.

CDN-induced type I IFN acts on DCs to boost NK cell activation and enhance antitumor effects

Type I IFN is a key modulator of DC function, promoting maturation and immune stimulatory functions (45, 46). We therefore hypothesized that CDN-induced type I IFN was acting in part on DCs, promoting NK cell effector function and enhanced tumor control. To determine whether type I IFN-dependent DC activation was important for NK cell activation, we used *Cd11c-Cre*, *Ifnar1*^{fl/fl} mice, in which *Ifnar1* undergoes deletion specifically in CD11c⁺ cells such as DCs (fig. S11). IFNAR1 expression was lost in most, but not all, CD11c⁺ MHC II⁺ cells in these mice (fig. S11). *Cd11c-Cre*, *Ifnar1*^{fl/fl} mice exhibited a partial defect in tumor rejection compared with *Ifnar1*^{fl/fl} (no Cre) control mice (Fig. 6A), indicating a role for type I IFN acting on DCs. The defect was modest, however, in comparison with the defect in *Ifnar1*^{-/-} mice or NK-depleted mice (Fig. 6A), consistent with type I IFN action on other cells, such as NK cells, as shown in Fig. 5. Relative to NK cells in *Ifnar1*^{fl/fl} (no Cre) control mice, NK cells in the tumor-draining lymph nodes of CDN-treated *Cd11c-Cre*, *Ifnar1*^{fl/fl} mice had lower levels of IFN-γ, granzyme B, and Sca-1 (Fig. 6B and fig. S12), indicating that type I IFN signaling on DCs is required for full NK cell activation. Again, however, the defect was only partial compared with PBS-treated control mice (Fig. 6B). Degranulation (CD107a) levels were similar between the two groups, suggesting that, for degranulation, the

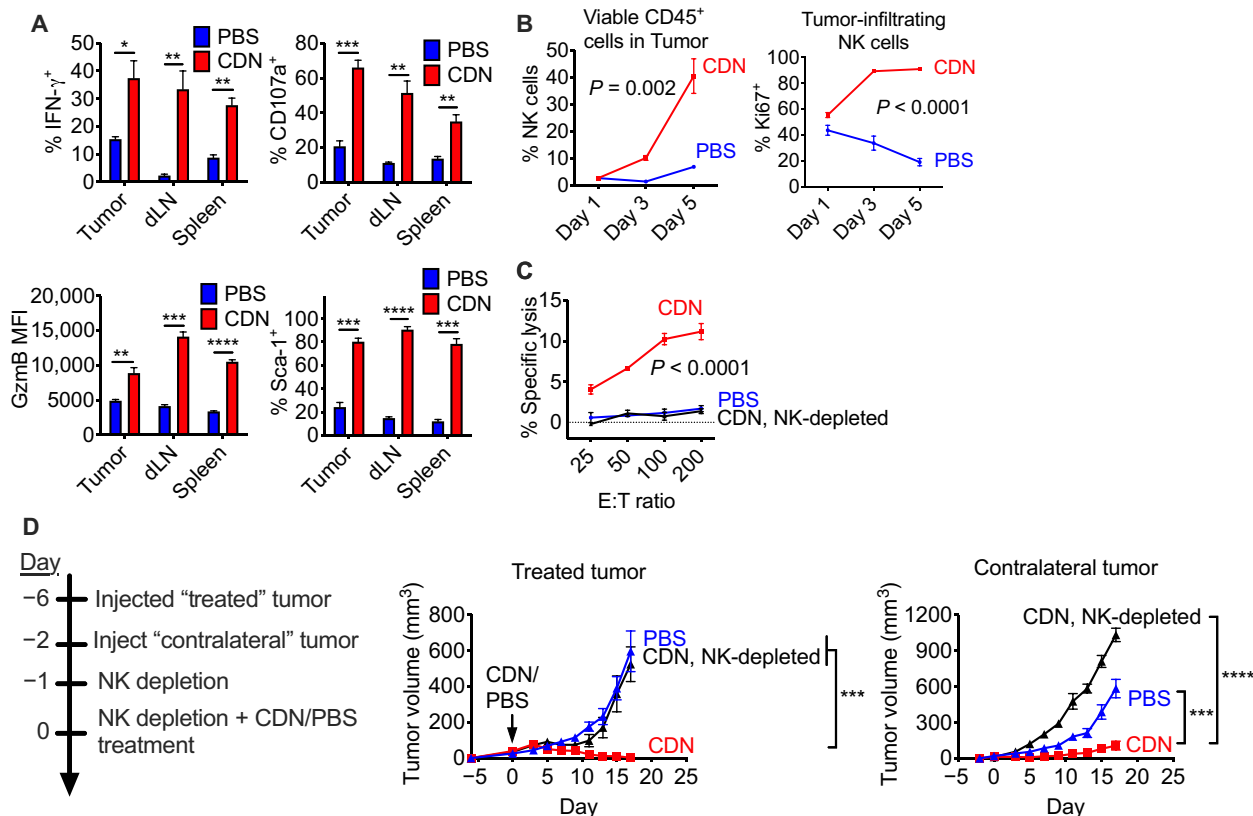


Fig. 3. Activation, proliferation, and cytotoxicity of NK cells induced by CDN treatments of tumors. (A) RMA-*B2m*^{-/-} tumors were established and treated as in Fig. 1B. Twenty-four hours later, tumors, tumor-draining LN (dLN), and spleens were harvested and stained for flow cytometry. NK cells were gated as viable, CD45⁺, CD3⁻, CD19⁻, F4/80⁻, Ter119⁻, NK1.1⁺, and Nkp46⁺ cells. *n* = 3. Two-tailed unpaired Student's *t* tests with the Holm-Sidak method for multiple comparisons were used. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. Data are representative of two independent experiments. MFI, mean fluorescence intensity. (B) RMA-*B2m*^{-/-} tumors were established, treated, harvested, stained, and analyzed on the days indicated. *n* = 3. Data are representative of two independent experiments and analyzed with two-way ANOVA. Error bars are shown but may be too small to see. (C) RMA-*B2m*^{-/-} tumors were established and treated as in Fig. 1B. Twenty-four hours after treatment, splenocytes were harvested and identical groups were pooled. Some groups were NK-depleted (see Materials and Methods). Cytotoxicity against RMA-*B2m*^{-/-} target cells was performed in technical triplicate, and error bars are shown but are typically too small to see. Data (representative of two independent experiments) were analyzed by two-way ANOVA. (D) Experimental schematic is shown. C1498-*B2m*^{-/-} tumors were established in both flanks of *Rag2*^{-/-} mice 4 days apart at a dose of 4×10^6 cells each. One day after the second, "contralateral," tumor was established, NK cells were depleted as in Materials and Methods. NK cells were depleted again the next day and weekly thereafter. Six days after the first, "treated," tumor was established, it was treated with PBS or 50 μ g of CDN. Tumor growth at both sites was monitored and analyzed as described in Fig. 1B. Data (combined from two independent experiments) were analyzed by two-way ANOVA. ****P* < 0.001, *****P* < 0.0001. *n* = 6 to 8.

direct action of type I IFN on NK cells may be more important than the indirect effects mediated by DCs. Splenocytes from CDN-treated *Cd11c-Cre, Ifnar1*^{fl/fl} mice also showed a small but reproducible reduction in ex vivo cytotoxicity against RMA-*B2m*^{-/-} tumor cells (*P* = 0.02) (Fig. 6C). Together, these data indicate that in CDN-treated tumors, type I IFN acts indirectly on DCs and directly on NK cells in promoting both NK cell activation and the rejection of tumors by NK cells.

Interleukin-15 is induced by CDN injections, dependent on type I IFN, and is important for the antitumor response

To address how type I IFN acts on DCs to enhance NK cell activation, we determined the impact of type I IFN on DC interleukin-15 (IL-15)–IL-15 receptor α (IL-15 α) expression after CDN treatments. Unlike many cytokines, IL-15 is trans-presented to cells: It associates with the IL-15 α chain during synthesis, and the IL-15/IL-15 α complex is presented to responding cells (47), where it binds the IL-2/15R β chain leading to signaling by the common γ chain, γ C. IL-15 signaling is especially important for NK cell biology because it enhances effector functions and promotes survival (48).

CDN-treated tumors had elevated levels of *Il15* and *Il15ra* transcripts relative to PBS-treated controls 24 hours after treatment (Fig. 7A). In parallel, cell surface IL-15 α expression was elevated in numerous cell types in the tumor-draining lymph node and spleen, including DCs, macrophages, monocytes, neutrophils, and NK cells (Fig. 7B and fig. S13). IFNAR1 blockade during CDN treatment inhibited the induction of *Il15* and *Il15ra* transcripts in tumors and cell surface IL-15 α expression on the aforementioned cell types (Fig. 7, A and B, and fig. S13). These data indicate that CDN treatment, mainly via the action of type I IFNs, induces IL-15/IL-15 α expression on numerous cell types in the tumor microenvironment and systemically.

When IL-15 was neutralized during CDN treatment, NK cells in the tumor-draining lymph nodes had significantly reduced IFN- γ , CD107a, granzyme B, and Sca-1 (Fig. 7C and fig. S14). In addition, neutralizing IL-15 caused a small but reproducible reduction in ex vivo cellular cytotoxicity mediated by splenocytes from the treated mice (*P* = 0.03) (Fig. 7D). Last, CDN-induced control of RMA-*B2m*^{-/-} tumors was markedly diminished in mice given

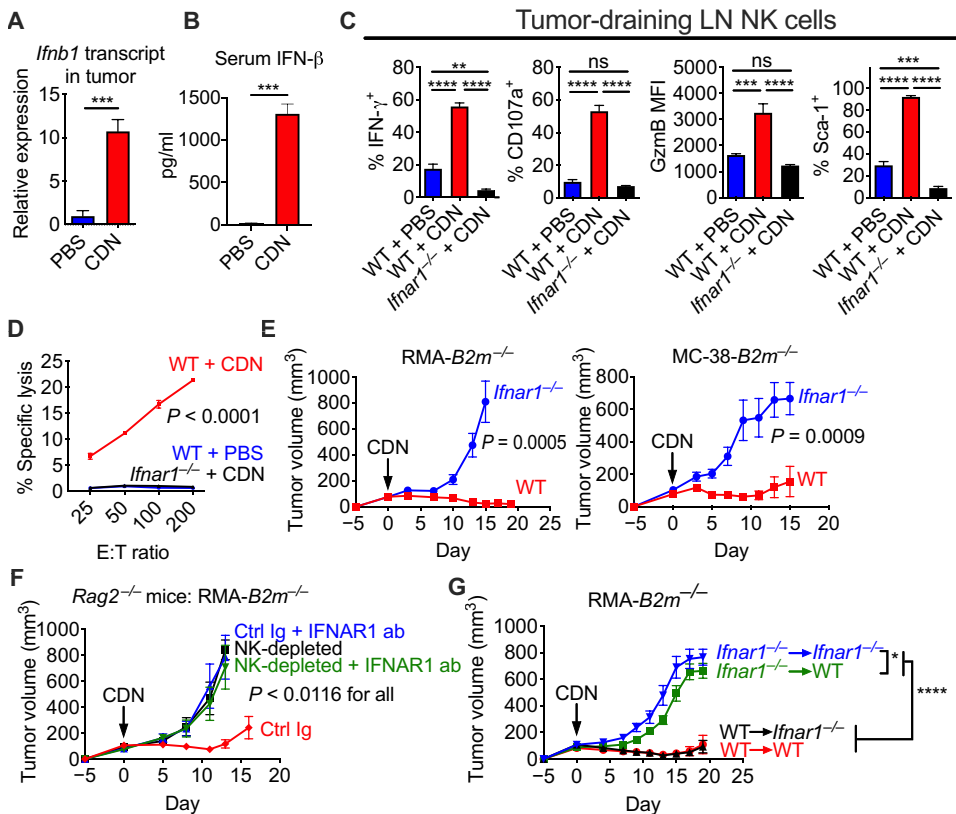


Fig. 4. Critical role for type I IFNs in the NK-dependent tumor rejection response induced by CDNs. (A) RMA-*B2m*^{-/-} tumors were established and treated as described in Fig. 1B. Twenty-four hours later, tumors were harvested, RNA was extracted, and quantitative reverse transcription PCR was performed to quantify *Ifnb1* transcripts. $n = 4$. $***P < 0.001$, as analyzed by two-tailed unpaired Student's *t* test. Data are representative of two independent experiments. (B) RMA-*B2m*^{-/-} tumors were established and treated as described in Fig. 1B. Six hours later, serum was collected and IFN- β was quantified by enzyme-linked immunosorbent assay. $***P < 0.001$, as analyzed by Mann Whitney test. Data are combined from two independent experiments. $n = 8$. (C) RMA-*B2m*^{-/-} tumors were established and treated as described in Fig. 1B. Twenty-four hours later, tumor-draining lymph node cells were harvested for flow cytometry analysis as in Fig. 3A. $n = 5$. $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$, as analyzed by one-way ANOVA with Tukey's correction for multiple comparisons. Data are representative of two independent experiments. (D) Cytotoxicity of splenocytes from tumor-bearing, PBS-, or CDN-treated mice analyzed as in Fig. 3C. Data are representative of two independent experiments. Error bars are shown but are typically too small to see. (E) Tumors were established in C57BL/6J or *Ifnar1*^{-/-} mice, treated, and analyzed as in Fig. 1B. $n = 5$ to 6. Data are representative of two independent experiments. (F) RMA-*B2m*^{-/-} tumors were established in *Rag2*^{-/-} mice and treated and analyzed as in Fig. 1B. Some animals were depleted of NK cells and/or given IFNAR1 neutralizing antibody (ab) (see Materials and Methods). Data are representative of two independent experiments. (G) Bone marrow chimeras were established with the indicated donor \rightarrow recipient combinations of C57BL/6J and *Ifnar1*^{-/-} bone marrow (see Materials and Methods). Eight weeks later, RMA-*B2m*^{-/-} tumors were established, treated, and analyzed as in Fig. 1B. $n = 8$ to 12 per group.

IL-15 neutralizing antibodies (Fig. 7E). Overall, the data suggest that CDNs induce IL-15 production and presentation, potentially by multiple cell types, in a type I IFN-dependent manner. The IL-15 then acts to boost NK cell effector function and tumor killing capacity, leading to greater tumor control in vivo.

DISCUSSION

The immunotherapeutic potential of NK cells for cancer, including solid cancers, has not been fully established. Our data demonstrate therapy-induced NK-dependent long-term remissions of several types of transplanted MHC I-deficient, CD8⁺ T cell-resistant,

solid tumors. The impressive impact of NK-dependent antitumor responses was not limited to MHC I-deficient tumors but also occurred in MHC I-high MC-38 tumors in *Rag2*^{-/-} mice, with some mice exhibiting long-term remissions. Like many tumor lines, MC-38 cells express abundant NKG2D ligands, and these cells are killed efficiently by NK cells in vitro (43), despite the high MHC I expression. It is likely that the key attribute predicting favorable NK-dependent effects induced by CDNs or other NK-mobilizing therapeutics is not solely MHC I deficiency but rather the overall sensitivity of the cells to NK cell killing, which reflects a balance of activating and inhibitory interactions (18).

Considering that most tumor cells express NKG2D ligands (49) or other ligands that activate NK cells (14) and are sensitive to NK killing in vitro, therapies that amplify NK cell activity have the potential to show efficacy in a broad variety of cancers, including many that are resistant to destruction by T cells. We propose that such therapies will complement therapies that mobilize T cell responses, including checkpoint therapies, by eliminating variants with antigen presentation defects. Therapeutic mobilization of NK cells may be especially important for tumors that lack strong T cell epitopes or have lost MHC expression, as well as for combining with therapeutic antibodies that mediate antibody-dependent cellular cytotoxicity mediated by NK cells.

STING agonists have shown marked efficacy in preclinical cancer models and are currently being tested in clinical trials. Most studies have focused on T cell-mediated responses induced by CDNs (32, 33, 35). In the present study, we demonstrated that intratumoral injection of CDNs triggered potent, NK-dependent, and CD8-independent re-

jection of several different NK-sensitive tumors originating from multiple tissue types. CDN injection triggered complete tumor rejection and long-term survival in a portion of the mice in most of the models tested.

Some of our studies used *B2m*^{-/-} tumor cells, which lack MHC I, and are therefore more sensitive to NK cells because they fail to engage inhibitory killer cell immunoglobulin-like receptors (or Ly49 receptors in mice) (16, 17). These models are potentially clinically relevant because many human tumors exhibit at least a partial loss of surface MHC I (5–7). Furthermore, resistance to checkpoint blockade correlates with the absence of tumor MHC I expression in some instances, with *B2M* mutations found among the nonresponding patient tumors (39–41).

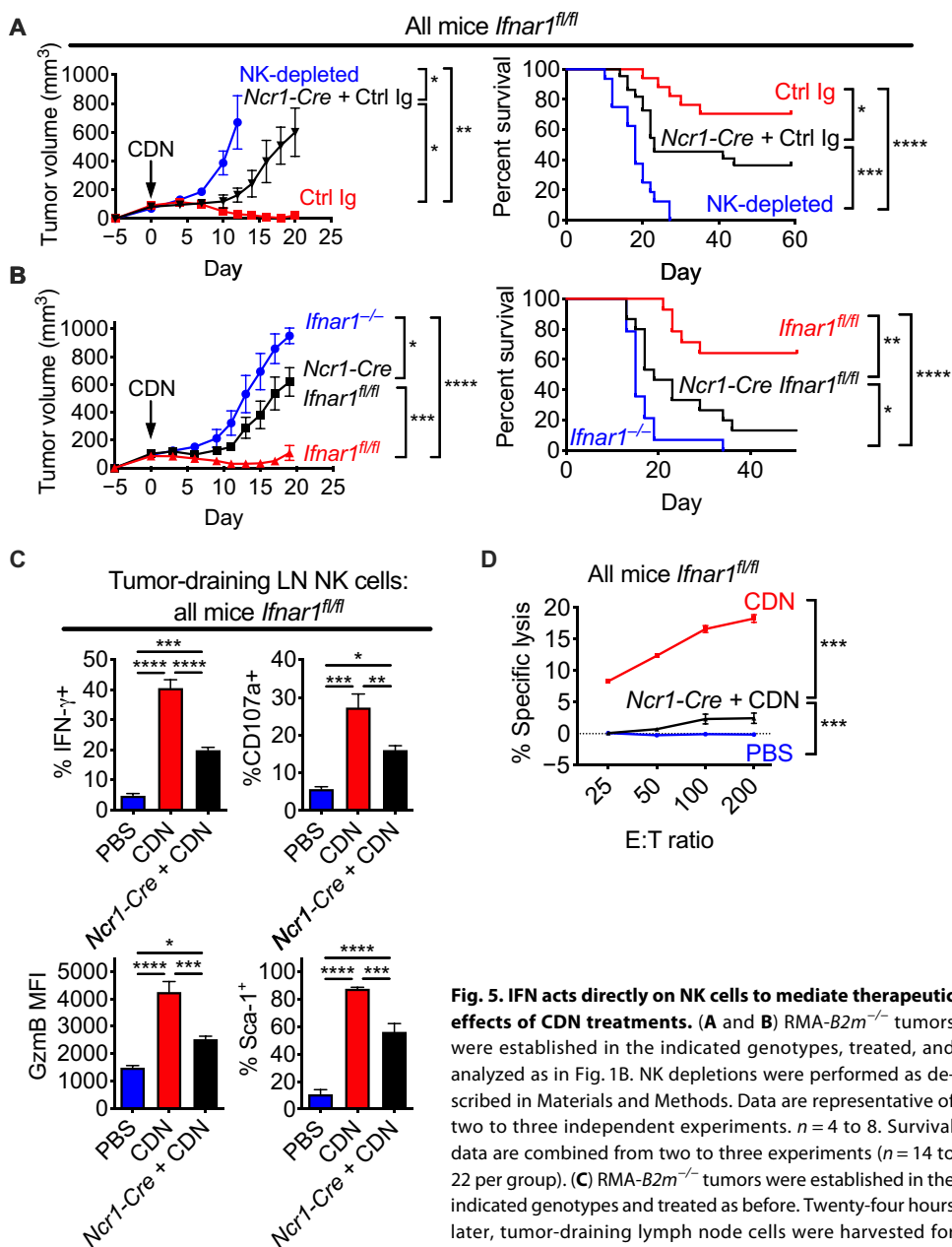


Fig. 5. IFN acts directly on NK cells to mediate therapeutic effects of CDN treatments. (A and B) RMA-B2m^{-/-} tumors were established in the indicated genotypes, treated, and analyzed as in Fig. 1B. NK depletions were performed as described in Materials and Methods. Data are representative of two to three independent experiments. $n = 4$ to 8. Survival data are combined from two to three experiments ($n = 14$ to 22 per group). (C) RMA-B2m^{-/-} tumors were established in the indicated genotypes and treated as before. Twenty-four hours later, tumor-draining lymph node cells were harvested for

flow cytometry as in Fig. 3A. $n = 4$ to 6. Data (representative of two independent experiments) were analyzed with one-way ANOVA with Tukey's correction for multiple comparisons. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. (D) Cytotoxicity of splenocytes from tumor-bearing PBS- or CDN-treated mice of the indicated genotypes analyzed as in Fig. 3C. Data are representative of two independent experiments. *** $P < 0.001$. Error bars are shown but are typically too small to see.

Other cancers, such as classical Hodgkin's lymphoma, generally have very low MHC I. The efficacy of PD-1 blockade in Hodgkin's lymphoma (6) may possibly be due to the activity of NK cells, given that NK cells express functional PD-1 in mouse tumor models (50).

Our results demonstrate that the CDN-induced, NK-mediated antitumor effects were dependent on type I IFN. A hallmark of STING activation is production of type I IFN, and many cell types, including both hematopoietic and nonhematopoietic cells, produce it in response to STING activation in tumors, including DCs, macrophages, monocytes, and endothelial cells. It has been shown that

cells in each of these compartments can contribute to the antitumor effects of intratumoral CDN injection (32, 34, 35, 51). Type I IFN enhances T cell responses (46) and is important for cancer immunosurveillance and the efficacy of cancer immunotherapies (38, 52, 53). Type I IFN is also important for NK cell biology (38), and NK cells from *Ifnar1*^{-/-} mice have greatly reduced cytotoxicity against tumor cell lines in vitro (38). Less clear is how type I IFN exerts its effects on NK cells, with reports of both direct and indirect actions. Consistent with direct action, mice lacking type I IFN signaling specifically in NK cells had reduced in vitro cytotoxicity against tumor cell lines (54). However, that study failed to find a survival difference between *Ncr1-Cre*, *Ifnar1*^{fl/fl} and *Ifnar1*^{fl/fl} control mice after exposure to oncogenic Abelson murine leukemia virus, leaving it unclear whether type I IFN acts directly on NK cells in the antitumor response.

Other reports have highlighted the importance of indirect action of type I IFN on NK cells, particularly cells of the myeloid lineage. DCs regulate NK cells both through direct interactions and the release of cytokines, such as IL-12, IL-15, and IL-18 (55). IL-15 is especially important for NK cell survival and homeostasis and is known to promote NK cell proliferation and effector activity (47, 56). Mice lacking *Il15ra* in either LysM- or CD11c-expressing cells exhibited substantial defects in NK cell homeostasis and activation (57). Type I IFN induces IL-15 production and presentation by DCs (55, 58), and it has been observed that IL-15 trans-presenting DCs are required for type I IFN-dependent "priming" of NK cells in vivo by TLR agonists or infections for enhanced ex vivo stimulation assays (58).

While informative, these studies conflicted in how type I IFN stimulates NK cells, and the role of type I IFN for NK cell-mediated tumor control in vivo remained unclear. Our findings provide clarity by indicating that both direct action of type I IFN and indirect action, via IL-15, are important for maximum NK cell antitumor activity in vivo. Our studies suggest that DCs are important contributors to indirect NK activation by type I IFN in vivo but do not rule out a role for other myeloid cell populations. We observed that other cell types up-regulated IL-15R after CDN treatments, including monocytes, macrophages, and even NK cells, suggesting that these other cell types may play some role in amplifying NK activity. We cannot test definitively whether the response depends on IL-15 from DCs with

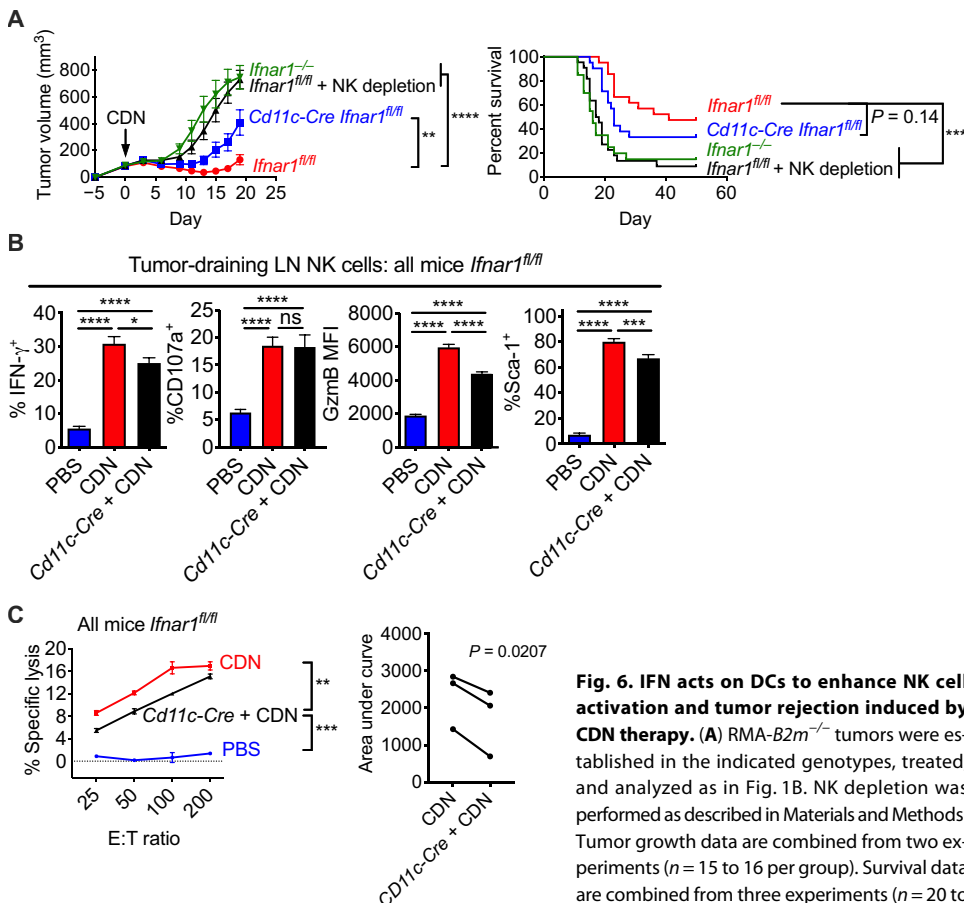


Fig. 6. IFN acts on DCs to enhance NK cell activation and tumor rejection induced by CDN therapy. (A) RMA-B2m^{-/-} tumors were established in the indicated genotypes, treated, and analyzed as in Fig. 1B. NK depletion was performed as described in Materials and Methods. Tumor growth data are combined from two experiments ($n = 15$ to 16 per group). Survival data are combined from three experiments ($n = 20$ to

21 per group). ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. (B) RMA-B2m^{-/-} tumors were established in the indicated genotypes and treated as before. Twenty-four hours later, flow cytometry analysis of tumor-draining lymph node NK cells was performed as in Fig. 3A. $n = 17$ to 22. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$ as analyzed with one-way ANOVA tests with Tukey's correction for multiple comparisons or Kruskal-Wallis with Dunn's multiple comparisons test for nonparametric data. Data are combined from four independent experiments. (C) Cytotoxicity of splenocytes from tumor-bearing PBS- or CDN-treated mice of the indicated genotypes was analyzed as in Fig. 3C. ** $P < 0.01$; *** $P < 0.001$. Error bars are shown but are typically too small to see. One experiment is shown in the left, and the reduced killing from *Cd11c-Cre, lfnar1*^{fl/fl} splenocytes was confirmed in a total of three independent experiments where the areas under the cytotoxicity curves were compared using paired, two-tailed Student's t tests (right).

available tools, because studies show that mice lacking IL-15 expression specifically in DCs (as well as those lacking IL-15 expression in macrophages) exhibit steady-state defects in NK cell numbers and functionality (57), making it impossible to attribute any phenotypes that we might observe to events occurring after establishing tumors and injecting CDNs. It has also been reported that tumors from patients with colorectal cancer have mutations in IL-15 and other cytokines and that this correlates with higher risk of tumor recurrence and decreased survival, suggesting that tumors may also be relevant sources of NK-activating cytokines (59). Our study found that type I IFN action on host cells, and not the tumor, was crucial for the antitumor effect in our model, but it remains possible that IFN induces IL-15 production by tumor cells in other cancers or models. Furthermore, we cannot rule out the importance of other CDN-induced, IFN-independent tumor-derived molecules. Last, we note that in addition to effects of CDNs on IL-15 (via IFN), CDNs are known to induce numerous other cytokines, chemokines, and cell surface receptors, and it is highly

likely that some of those other induced molecules also play important roles in the antitumor NK response.

CDN treatment led to systemic activation of NK cells and delayed the growth of distal tumors. Intratumoral injections of CDN led to increased levels of IFN- β in the serum, and it is likely that the systemic type I IFN response promoted the systemic NK cell activation and antitumor effects. There are, however, other potential mechanisms of systemic NK cell activation that may play some role. When very high doses of CDNs are injected in mice with two tumors, some leakage from the injected tumor occurs, and low amounts of CDNs can be detected in distal tumors (32). Although we used much lower doses of CDN than in that study, it remains possible that CDN leakage from the tumors into the circulation contributed to the systemic NK cell activation that we observed. The possibility that large numbers of NK cells that were initially activated locally near the tumor recirculated to the spleen and to distal tumors appears less likely given that such a large percentage of splenic NK cells were activated shortly after local CDN administration. Regardless of the exact mechanism, our data make clear that intratumoral CDN treatment alone is capable of promoting antitumor effects on distal tumors independently of T cells.

Cancer immunotherapy, especially checkpoint blockade, has led to major improvements in cancer treatment (1, 2), and although substantial numbers of long-term remissions have been achieved in several cancers, many patients do not respond.

Combining checkpoint therapy with CDN therapy may be beneficial not only because CDNs amplify T cell responses (32, 35) but also because tumor cells in patients treated with checkpoint inhibitors are sometimes selected for loss of MHC I (39–41), and CDN-activated NK cells may eliminate those cells. Furthermore, NK cells in tumors express checkpoint receptors such as PD-1 and T cell immunoreceptor with Ig and ITIM domains (TIGIT) (50, 60), suggesting that checkpoint therapy could enhance the function of CDN-activated NK cells. Combinations of CDNs with NK-activating cytokines such as IL-15, IL-2, IL-12, and IL-18 may also provide added benefit (61, 62). Last, blocking endogenous interactions that lead to NK cell desensitization (63, 64) or providing CDNs in combination with antibodies that mediate NK-dependent antibody-dependent cellular cytotoxicity of cancer cells may also be impactful.

In conclusion, our results show that CD8⁺ T cell-resistant tumors can be effectively treated using CDNs. The antitumor effects were mediated by NK cells and dependent on type I IFN, which

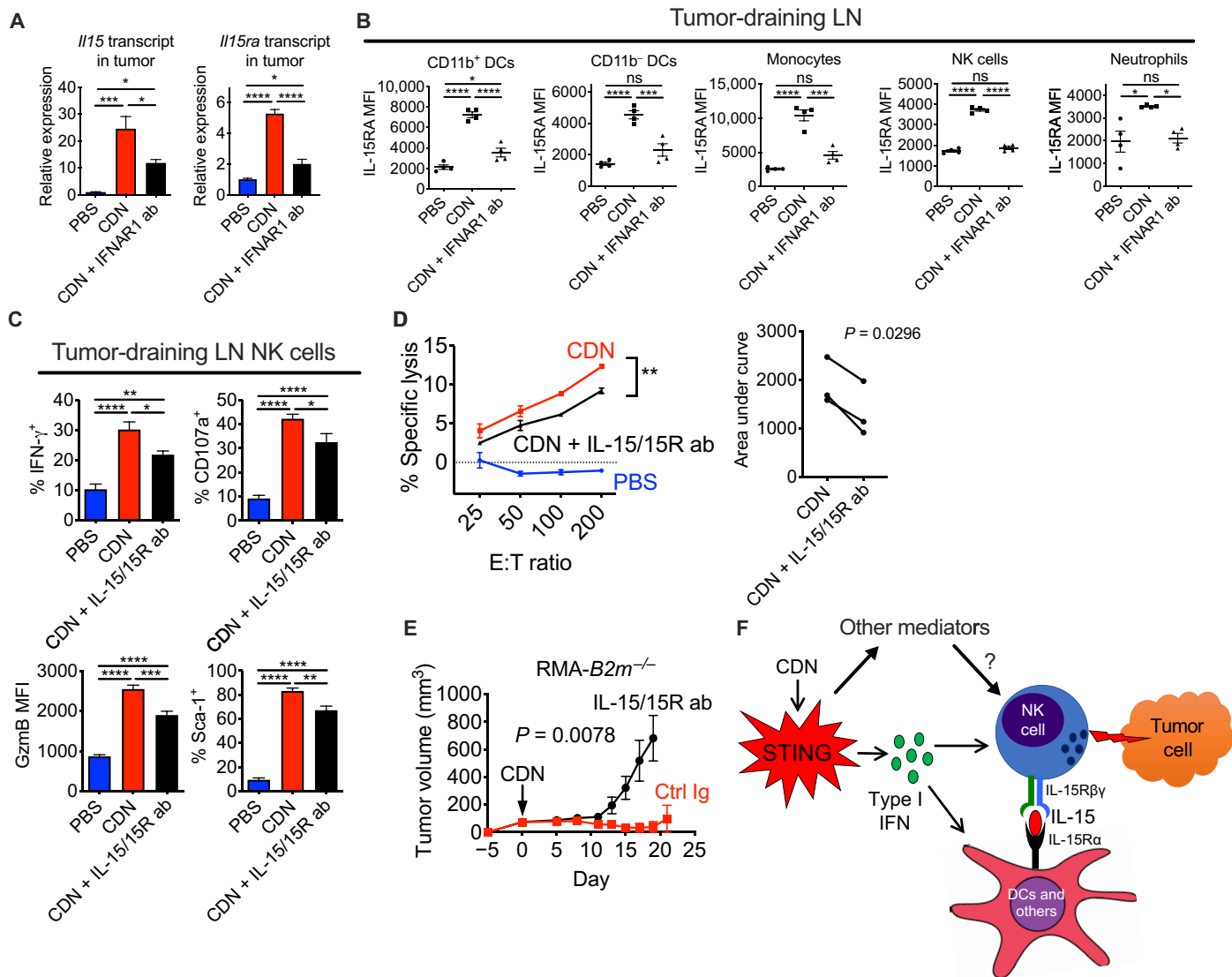


Fig. 7. IL-15/IL-15R α expression is induced on DCs and other cells by IFNs after CDN therapy and contributes significantly to optimal NK cell activation and tumor rejection. (A) RMA-B2m^{-/-} tumors were established, treated, RNA-extracted, and analyzed by quantitative PCR for *Il15* or *Il15ra* transcripts as in Fig. 4A. Some mice received IFNAR1 neutralizing antibody (see Materials and Methods). *n* = 4. Data (representative of two independent experiments) were analyzed with one-way ANOVA with Tukey's correction for multiple comparisons. (B) RMA-B2m^{-/-} tumors were established and treated as before. Twenty-four hours later, tumor-draining lymph node cells were harvested for flow cytometry as in Materials and Methods. The mean fluorescence intensity of IL-15RA (BAF551) is displayed. Viable CD3⁺, CD19⁻, Ter119⁻ cells were further gated on DCs (NK1.1⁻, Ly6G⁻, CD11c^{high}, and MHC-II^{high}), monocytes (NK1.1⁻, Ly6G⁻, CD11b^{high}, and Ly6C^{high}), neutrophils (NK1.1⁻, CD11b⁺, and Ly6G⁺), NK cells (NK1.1⁺), and macrophages (NK1.1⁻, Ly6G⁻, CD11b⁺, and F4/80⁺). *n* = 4. **P* < 0.05; *****P* < 0.001; *****P* < 0.0001, as analyzed by one-way ANOVA with Tukey's correction for multiple comparisons. Data are representative of two independent experiments. (C) RMA-B2m^{-/-} tumors were established and treated, and tumor-draining lymph node NKs were analyzed by flow cytometry as in Fig. 3A. Some mice received IL-15/IL-15R neutralizing antibody (see Materials and Methods). *n* = 5. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. Data (representative of two independent experiments) were analyzed with one-way ANOVA with Tukey's correction for multiple comparisons. (D) Cytotoxicity of splenocytes from tumor-bearing PBS- or CDN-treated mice was analyzed as in Fig. 3C. Some mice received IL-15/IL-15R neutralizing antibody or control Ig (see Materials and Methods). ***P* < 0.01. Error bars shown but typically too small to see. One experiment is shown in the left, and the reduced killing from IL-15R neutralization was confirmed in a total of three independent experiments where the areas under the cytotoxicity curves were compared using paired, two-tailed Student's *t* tests (right). (E) RMA-B2m^{-/-} tumors were established, treated, and analyzed as in Fig. 1B. Mice received 5 μ g of IL-15/IL-15R antibody or control IgG (see Materials and Methods). *n* = 5 per group. Data are representative of two independent experiments. (F) Model of CDN-induced NK cell activation. Intratumoral CDN treatment activates the STING pathway, resulting in production of type I IFN and other mediators including cytokines and chemokines, boosting NK cell effector functions and antitumor activities. Type I IFN elicits its effects on NK cells by direct action and indirectly via DCs, which up-regulate IL-15/IL-15R α complexes to enhance NK cell antitumor effects.

boosts NK cell antitumor responses in vivo. Mechanistically, type I IFN boosts NK cell responses by both direct action and indirect action via DCs, which induce IL-15 to further promote NK activa-

tion and tumor destruction (Fig. 7F). These findings support the view that NK cells could be a cornerstone of next-generation cancer immunotherapies.

MATERIALS AND METHODS**Study design**

The objectives of this work were to examine the mechanisms of NK cell-mediated antitumor effects after intratumoral CDN treatment of MHC I-deficient and MHC I-expressing tumors. For our studies, tumors were established subcutaneously in mice, PBS or CDNs were injected intratumorally, and tumor growth, overall survival, and NK cell activation status were recorded. Male and female mice were equally used, and experimental groups/treatments were randomized among mice in the same cage when possible. In general, experimental groups consisted of at least 5 to 6 mice, but in some experiments, up to 12 were used. We did not use a power analysis to calculate sample size and did not exclude data. All experiments were performed at least twice, and in some cases, experiments were pooled. The investigators were not blinded.

Mouse strains

Mice were maintained at the University of California, Berkeley. C57BL/6J, CD45.1-congenic (B6.SJL-*Ptprca*^a *Pepcb*^b/BoyJ), *Rag2*^{-/-}, *Rag2*^{-/-} *Il2rg*^{-/-}, *Ifnar1*^{-/-} (all on the B6 background), and BALB/cJ mice were purchased from the Jackson Laboratory. *Ncr1*^{iCre} and *Sting*^{gt/gt} mice on the B6 background were gifts from E. Vivier and R. Vance, respectively. NK-DTA mice were generated by breeding *Ncr1*^{iCre} mice to B6-*Rosa26*^{LSL-DTA} mice (Jackson Laboratory). *Ncr1*^{iCre/+}, *Ifnar1*^{fl/fl} and *CD11c-Cre*, *Ifnar1*^{fl/fl} mice, all on the B6 background, were generated by breeding *Ncr1*^{iCre} and *CD11c* (*Itgax*)-*Cre-eGFP* (Jackson Laboratory) mice to *Ifnar1*^{fl/fl} mice (Jackson Laboratory). All mice used were aged 8 to 30 weeks. All experiments were approved by the University of California (UC) Berkeley Animal Care and Use Committee.

Cell lines and culture conditions

RMA (obtained from M. Bevan, who received it from K. Karre, Karolinska Institute, Stockholm, Sweden), CT26 (obtained from Aduro Biotech), 4T1 (obtained from R. Weinberg), and C1498 (purchased from American Type Culture Collection) were cultured in RPMI 1640 (ThermoFisher Scientific). B16-F10 (obtained from the UC Berkeley Cell Culture Facility) and MC-38 (obtained from J. Allison) were cultured in Dulbecco's modified Eagle's medium (ThermoFisher Scientific). In all cases, media contained 5% fetal bovine serum (FBS) (Omega Scientific), glutamine (0.2 mg/ml; Sigma-Aldrich), penicillin (100 U/ml; ThermoFisher Scientific), streptomycin (100 µg/ml; ThermoFisher Scientific), gentamycin sulfate (10 µg/ml; Lonza), 50 µM β-mercaptoethanol (EMD Biosciences), and 20 mM Hepes (ThermoFisher Scientific) and the cells were cultured in 5% CO₂. *B2m*^{-/-} cell lines were generated using CRISPR-Cas9 (described below). All cells tested negative for mycoplasma contamination.

Generation of cell lines using CRISPR-Cas9

Plasmids containing Cas9 and *B2m*-targeting guide sequence were generated previously (62). The *Ifnar1*-targeting CRISPR-Cas9 plasmid was generated by cloning the guide sequence (GCTGGTG-GCCGGGGCGCCTT) into PX330 (Addgene) following the recommended protocol. To generate knockout cell lines, plasmids were transiently transfected using either Lipofectamine 2000 (ThermoFisher Scientific) (CT26, 4T1, B16-F10, and MC-38) or by nucleofection (RMA and C1498) (Kit T, Lonza). One week later, MHC I- or IFNAR1-deficient cells were sorted using a FACSAria cell sorter. For B16-F10, cells were incubated with IFN-β (100 ng/ml; BioLegend)

overnight before sorting to easily distinguish MHC I⁺ and MHC I⁻ cells.

In vivo tumor growth experiments

Cells were washed and resuspended in PBS (ThermoFisher Scientific), and 100 µl containing 4 × 10⁶ cells were injected subcutaneously. Tumor growth was measured using calipers, and tumor volume was estimated using the ellipsoid formula: $V = (\pi/6)ABC$. In some experiments, mice were NK-depleted by intraperitoneal injection of 250 µg of anti-NK1.1 (clone PK136, purified in our laboratory) or 10 µl of anti-asialo-GM1 (BioLegend) for C57BL/6J and BALB/c mice, respectively. Mice were CD8- and CD4-depleted by intraperitoneal injection of 250 µg of anti-CD8b.2 (clone 53.5.8, Leinco) or 250 µg of anti-CD4 (clone GK1.5, Leinco), respectively. Whole rat immunoglobulin G (IgG; Jackson ImmunoResearch) was used as a control. Depleting or control antibodies were injected 2 days and 1 day before tumor inoculation and continued weekly thereafter. Depletions were confirmed by flow cytometry. Five days after tumor inoculation, when tumors were ~50 to 150 mm³, mice were injected intratumorally with PBS or 1 × of 50 µg of c-di-AMP (RMA-*B2m*^{-/-}, B16-F10-*B2m*^{-/-}, C1498-*B2m*^{-/-}, and MC38-*B2m*^{-/-}) or 3 × of 25 µg of c-di-AMP (CT26-*B2m*^{-/-} and 4T1-*B2m*^{-/-}) in a total volume of 100 µl (PBS). In some experiments, mice received 500 µg of anti-IFNAR1 (clone MAR1-5A3, Leinco), 200 µg of anti-TNF-α (clone TN3-19.12, Leinco), or control rat IgG intraperitoneally on day -1, on day 0, and again on days 1 and 4. In some experiments, mice received 5 µg of anti-IL-15/15R (clone GRW15PLZ, ThermoFisher Scientific) or control rat IgG intraperitoneally on day -1, once again intratumorally mixed with CDN on day 0, and again intraperitoneally on days 1 and 2.

Flow cytometry

Single-cell suspensions of spleens and lymph nodes were generated by passing cells through a 40-µm filter. Red blood cells were removed from spleens using ammonium-chloride-potassium (ACK) lysing buffer (made in our laboratory). Tumors were chopped with a razor blade and dissociated in a gentleMACS Dissociator (Miltenyi) before passage through an 80-µm filter. For assessing NK activation, cell suspensions were incubated for 4 hours in medium containing brefeldin A (BioLegend), monensin (BioLegend), and anti-CD107a antibodies before surface and intracellular staining. LIVE/DEAD stain (ThermoFisher Scientific) was used to exclude dead cells. FcγRII/III receptors were blocked with the 2.4G2 hybridoma supernatant (prepared in the lab). Staining with fluorochrome- or biotin-conjugated antibodies occurred at 4°C for 30 min in fluorescence-activated cell sorting buffer (2.5% FBS and 0.02% sodium azide in PBS). When necessary, fluorochrome-conjugated streptavidin was added. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and stained with fluorochrome-conjugated antibodies for 30 min at 4°C in Perm/Wash buffer (BD Biosciences). Flow cytometry was performed using an LSRFortessa or an LSRFortessa X-20 (BD Biosciences). Data were analyzed with FlowJo (Tree Star).

Antibodies

For flow cytometry, we used the following antibodies: anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD3ε (145-2C11), anti-CD4 (GK1.5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (6D5), anti-F4/80 (BM8), anti-Ly6C (HK1.4), anti-Ly6G (1A8), anti-NKp46 (29A1.4), anti-NK1.1 (PK136), anti-Sca-1 (D7), anti-Ter119 (TER-119), anti-Ki67 (Sola15), anti-CD107a (1D4B),

anti-I-A/I-E (M5/114.15.2), anti-IFN- γ (XMG1.2), and anti-IFNAR1 (MAR1-5A3) (all from BioLegend); anti-H-2K^b (AF6-88.5) and anti-granzyme B (GB11) (both from BD Biosciences); anti-IL-15R α (BAF551) (R&D Systems).

Ex vivo cytotoxicity assay

Cytotoxicity by splenocytes was assessed with a standard 4-hour ⁵¹Cr-release assay. About 24 hours after CDN or PBS treatment of tumors, spleens were harvested and treated with ACK lysing buffer. Pooled splenocytes from four to six mice were used as effector cells. Triplicate samples of 10⁴ ⁵¹Cr-labeled RMA-B2m^{-/-} cells per 96-well V-bottom plate well were incubated with splenocytes at the indicated E:T ratios for 4 hours before determining the percent ⁵¹Cr release in the supernatant. % Specific lysis = 100 × (experimental – spontaneous release_{Avg}) / (maximum release_{Avg} – spontaneous release_{Avg}), where maximum release was release with addition of Triton X-100 (final concentration, 2.5%).

Where shown, pooled splenocytes were NK-depleted by incubating on ice for 30 min with anti-NKp46-biotin (BioLegend) and anti-NK1.1-biotin (BioLegend), followed by 20-min incubation with streptavidin magnetic beads (BioLegend), and magnetic removal of bead-bound cells. Depletion (>95%) was confirmed by flow cytometry.

RNA isolation, reverse transcription, and quantitative polymerase chain reaction

Tumors were harvested and dissociated using the gentleMACS dissociator (Miltenyi), and total RNA was isolated using the RNeasy Mini Kit (Qiagen) and treated with deoxyribonuclease I (Qiagen). Complementary DNA (cDNA) was generated using the iScript reverse transcription kit (Bio-Rad). Quantitative real-time polymerase chain reaction (PCR) was performed using SsoFast EvaGreen Supermix (Bio-Rad) with 25 ng of cDNA per reaction in a CFX96 Thermocycler (Bio-Rad). *Gapdh* and *Ubc* were used as references.

Primer sequences were as follows: *Gapdh*, TGTGTCCGTCGTGGATCTGA (forward) and TTGCTGTTGAAGTCGCAGGAG (reverse); *Ubc*, GCCCAGTGTACCACCAAGA (forward) and CCCATCACACCCAAGAACA (reverse); *Ifnb1*, ATGAACTC-CACCAGCAGACAG (forward) and ACCACCATCCAGGCG-TAGC (reverse); *Il15*, GTGACTTTCATCCCAGTTGC (forward) and TTCCTTGAGCCAGATTCTG (reverse); and *Il15ra*, CCCA-CAGTTCAAAATGACGA (forward) and GCTGCCTTGATTT-GATGTACCAG (reverse).

Enzyme-linked immunosorbent assay

Tumors of ~100 mm³ were injected with PBS or 50 μ g of CDN. Six hours later, serum was harvested and IFN- β was quantified by enzyme-linked immunosorbent assay (BioLegend) following the manufacturer's instructions.

Bone marrow chimeras

Recipient mice were irradiated with 10 Gy (5 Gy + 5 Gy on consecutive days), followed by intravenous injection of 10⁷ donor bone marrow cells suspended in 100 μ l of PBS. WT mice were B6-CD45.1, and the *Ifnar1*^{-/-} donors were on the B6 background (CD45.2). After 8 weeks, chimerism was assessed by staining blood cells for CD45.1 and CD45.2 expression and analyzing by flow cytometry, followed by use of the mice in experiments.

Statistics

Statistics were performed using Prism (GraphPad). For tumor growth and survival, two-way analysis of variance (ANOVA) and log-rank (Mantel-Cox) tests were used. For NK activation and quantitative PCR, unpaired two-tailed Student's *t* tests or one-way ANOVA followed by Tukey's multiple comparisons tests were used when data fit a normal distribution. For nonparametric data, the Kruskal-Wallis test with Dunn's multiple comparisons was used. Two-way ANOVA was used for cytotoxicity, and in some instances, the areas under the curves were compared using paired two-tailed Student's *t* tests. Significance is indicated as follows: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

SUPPLEMENTARY MATERIALS

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Fig. S1. MHC I expression and growth of B2m^{-/-} tumor cell lines.

Fig. S2. Verifying in vivo depletions.

Fig. S3. Representative flow plots for Fig. 3A.

Fig. S4. Representative flow plots for Fig. 3B.

Fig. S5. Systemic T cell-independent antitumor effects of CDNs in B16-F10-B2m^{-/-}.

Fig. S6. Representative flow plots for Fig. 4B.

Fig. S7. IFNAR1 neutralization prevents CDN-induced NK cell activation, cytotoxicity, and tumor rejection.

Fig. S8. Bone marrow chimera reconstitution efficiency.

Fig. S9. NK cell and T cell IFNAR1 expression in *Ncr1-iCre, Ifnar1^{fl/fl}* mice.

Fig. S10. Representative flow plots for Fig. 5A.

Fig. S11. IFNAR1 expression by DCs and NK cells in *CD11c-Cre, Ifnar1^{fl/fl}* mice.

Fig. S12. Representative flow plots for Fig. 6B.

Fig. S13. IFNAR1 neutralization reduces CDN-induced IL-15RA expression.

Fig. S14. Representative flow plots for Fig. 7C.

Table S1. Raw data file (in Excel spreadsheet).

[View/request a protocol for this paper from Bio-protocol.](#)

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NK cells mediate clearance of CD8 T cell–resistant tumors in response to STING agonists

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Priming NK Cells for Tumor Destruction

Tumors with low neoantigen burden and/or diminished class I MHC expression evade CD8 T cells, but NK cells provide another option to target such tumors for immune elimination. Nicolai *et al.* used several mouse models to investigate the mechanisms by which intratumoral injection of a cyclic dinucleotide (CDN) agonist for STING potentiated the antitumor activity of NK cells, both in the injected tumor and at a remote, uninjected tumor site. CDN administration induced type I interferons that directly promoted NK cell activation and simultaneously enabled an indirect pathway of activation driven by induction of IL-15 and IL-15R α on dendritic cells. These findings provide preclinical evidence that amplification of NK-based tumor immunity may offer a valuable adjunct to immunotherapy approaches promoting CD8 T cell–dependent antitumor responses.

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