Neoadjuvant STING Activation, Extended Half-life IL2, and Checkpoint Blockade Promote Metastasis Clearance via Sustained NK-cell Activation

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ABSTRACT

Combination immunotherapy treatments that recruit both innate and adaptive immunity have the potential to increase cancer response rates by engaging a more complete repertoire of effector mechanisms. Here, we combined intratumoral STimulator of INterferon Genes (STING) agonist therapy with systemically injected extended half-life IL2 and anti–PD-1 checkpoint blockade (hereafter CIP therapy) to drive innate and adaptive antitumor immunity in models of triple-negative breast cancer. Unlike treatment with the individual components, this trivalent immunotherapy halted primary tumor progression and led to long-term remission for a majority of animals in two spontaneously metastasizing orthotopic breast tumor models, though only as a neoadjuvant therapy but not adjuvant therapy. CIP therapy induced antitumor T-cell responses, but protection from metastatic relapse depended on natural killer (NK) cells. The combination of STING agonists with IL2/anti–PD-1 synergized to stimulate sustained granzyme and cytokine expression by lung-infiltrating NK cells. Type I IFNs generated as a result of STING agonism, combined with IL2, acted in a positive-feedback loop by enhancing the expression of IFNAR-1 and CD25 on lung NK cells. These results suggest that NK cells can be therapeutically targeted to effectively eliminate tumor metastases.

Introduction

Combination treatments driving synergistic pathways of immunity against tumors may enhance cancer immunotherapy outcomes. Therapies promoting innate and adaptive immune responses in tandem may be of particular value by leveraging the full armamentarium of the immune response (1, 2). For example, natural killer (NK) cells have been shown to engage in cross-talk with dendritic cells (DC) through tumor-specific mAbs; antibodies bound to tumor cells trigger antibody-dependent cellular cytotoxicity from NK cells followed by immune complex uptake by DCs for T-cell priming (3, 4). In addition to direct cytolytic effects, NK cells secrete proinflammatory cytokines such as IFNγ and TNFα, which induce antiproliferative and antiangiogenic effects within the tumor microenvironment and further stimulate T-cell cytotoxicity (5).

We previously reported on a combination therapy, AIPV, comprised of a peptide vaccine, tumor-specific antibody, extended half-life IL2 (an albumin-IL2 fusion, Alb-IL2), and anti–PD-1 checkpoint blockade (6). Alb-IL2 has a terminal half-life around 50 hours in circulation (7). Weekly dosing with this quaternary combination leads to rejection of large, established tumors and generates long-lived T-cell memory responses in several syngeneic cancer models, including a genetically engineered model of melanoma. These therapeutic responses are dependent on multiple innate immune cell populations, including NK cells, macrophages, and neutrophils. However, the use of two tumor antigen–specific components (vaccine and antibody) makes AIPV therapy challenging to translate to clinical testing. We thus sought to identify a simplified, tumor-agnostic combination treatment capable of similarly potentiating innate and adaptive immune responses in tandem.

The antitumor antibody and vaccine components of AIPV drive initial rapid tumor cell killing and antitumor T-cell priming, respectively. We hypothesized that intratumoral treatment with ligands for STimulator of INterferon Genes (STING) could replace these functions. STING signaling has been implicated in the generation of tumor debris through immunogenic cell death (8–10). Following tumor antigen release, STING signaling can trigger DC activation and cross-presentation (10). Exploiting this effect, STING agonists have been explored as vaccine adjuvants for improving T-cell priming (11, 12). Notably, STING expression in host cells, rather than cancer cells, is essential for the generation of tumor-reactive cytotoxic T cells in many models of cancer treatment (13). Furthermore, STING activation also stimulates innate immunity, and STING agonists have been explored for their ability to stimulate natural killer (NK) cells in CD8+ T-cell–resistant preclinical tumor models (14, 15). Tmem173 (STING) gene expression is also associated with tumoricidal capacity of neutrophils in breast cancer (16).

Encouraged by the precedent of STING agonists serving as pleiotropic modulators of both adaptive and innate immune cells, we explored the use of intratumorally injected STING agonists (cyclic dinucleotides, C) as a means to promote antitumor immunity in...
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concert with systemic administration of Alb-IL2 (I) and anti–PD-1 (P) in orthotopic mouse models of triple-negative breast cancer. Naturally metastatic syngeneic breast tumor models allow for profiling the immune response directed against primary tumors as well as distant metastases, from which patients with triple-negative breast cancer are more likely to suffer than other patients with breast cancer (17). Although CIP therapy elicited CD8+ T-cell priming, we found that in the setting of neoadjuvant treatment, this combination therapy also triggered activation of NK cells that mediated protection from metastases in the lungs. Type I IFNs elicited from STING activation in the primary tumor, together with IL2, drove sustained proliferation and expression of effector molecules, including granzyme B, IFNγ, TNFα, perforin, and NKG2D in lung NK cells. Hence, this tumor-agnostic immunotherapy elicited combined innate and adaptive immune responses, with the innate response playing an important role in protection from metastases in multiple models of breast cancer.

Materials and Methods

Mice

BALB/c, C57BL/6, NOD.Cg-PtkdcΔcdΔJ (NOD SCID), NOD.Cg-PtkdcΔcdΔJ E0771 cells were inoculated into the fourth mammary fat pad of BALB/c, NOD SCID, or NSG mice, and 5 × 10^7 E0771 cells were inoculated into the fourth mammary fat pad of C57BL/6 mice. Treatment was administered according to the referenced timelines, generally beginning when tumors reached 50 to 100 mm^3 (0.5 L/1.1m^3). Nine days after the start of treatment, mice were anesthetized with isoflurane and provided preputial subcutaneous sustained-release buprenorphine (1 mg/kg, ZooPharm) and meloxicam (5 mg/kg). The primary tumor and draining inguinal lymph node were surgically removed and the wound was closed with surgical clips. Inguinal lymph nodes were removed because of primary tumors frequently growing around the node; therefore, the inguinal lymph node was removed in all mice. Meloxicam (5 mg/kg) was dosed every 24 hours for 3 days postoperatively. Mice were then monitored every other day and euthanized if signs of distress, >20% body weight loss, or poor body condition were observed.

Biotumors for orthotopic studies, 5 × 10^6 E0771-Luc tumor cells were inoculated into the fourth mammary fat pad of BALB/c, NOD SCID, or NSG mice, and 5 × 10^6 E0771 cells were inoculated into the fourth mammary fat pad of C57BL/6 mice. Treatment was administered according to the referenced timelines, generally beginning when tumors reached 50 to 100 mm^3 (0.5 L/1.1m^3). Nine days after the start of treatment, mice were anesthetized with isoflurane and provided preputial subcutaneous sustained-release buprenorphine (1 mg/kg, ZooPharm) and meloxicam (5 mg/kg). The primary tumor and draining inguinal lymph node were surgically removed and the wound was closed with surgical clips. Inguinal lymph nodes were removed because of primary tumors frequently growing around the node; therefore, the inguinal lymph node was removed in all mice. Meloxicam (5 mg/kg) was dosed every 24 hours for 3 days postoperatively. Mice were then monitored every other day and euthanized if signs of distress, >20% body weight loss, or poor body condition were observed.

Bioluminescence imaging

4T1-Luc tumor-bearing mice were monitored postoperatively for development of metastases using bioluminescence imaging beginning 1 week after surgical resection of the primary tumor. Animals were injected intraperitoneally with 150 mg/kg sterile filtered 48 rat IgG2a (2A3), control rat IgG2b (LTF2) were purchased from BioXCell. Anti-mouse CD8 (2.43), anti-mouse Ly6G (1A8), anti-mouse CD4 (GK1.5), anti-mouse NK1.1 (PK136), anti-mouse IFNAR-1 (MAR1–5A3), control rat IgG2a (2A3), and control rat IgG2b (LTF2) were purchased from BioXCell. Anti–PD–1 was administered intraperitoneally at 200 μg per dose on the indicated schedules. Cellular depletions were conducted by administering 400 μg of depleting antibody (2.43, 1A8, GK1.5, PK136) or isotype control (2A3, LTF2) intraperitoneally beginning 1 day prior to initiation of therapy then every 3 days for a total of seven doses. Anti-asialoGM1 (Wako Chemicals) was administered intraperitoneally in 50 μL doses for NK-cell depletion on the same schedule. IFNAR-1 blocking was accomplished by administering 500 μg of MAR1-5A3 antibody intraperitoneally 48 and 24 hours prior to commencement of therapy and dosed every 3 days.

Aspartate aminotransferase assay and alanine transaminase assay

4T1-Luc tumor-bearing mice were treated with 30 μg CDN i.t., 30 μg Alb-IL2 i.p., and 200 μg anti–PD–1 i.p., or listed subsets beginning 8 days after tumor inoculation. A total of 50 μL of blood was sampled 24, 48, and 72 hours after treatment initiation and assayed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity according to the manufacturer’s protocol (Sigma).

Evans blue pulmonary vessel permeability assay

4T1-Luc tumor-bearing mice were treated with 30 μg CDN i.t., 30 μg Alb-IL2 i.p., and 200 μg anti–PD–1 i.p., or listed subsets beginning 8 days after tumor inoculation. A total of 100 μg recombinant human IL2 (Peprotech) was administered intraperitoneally every 12 hours for three doses as a positive control. A total of 24 hours after one round of treatment, animals were injected intravenously with 200 μL of 0.5% Evans blue dye. Lungs were harvested 30 minutes after dye injection, weighed, and placed in 500 μL formamide. Lung samples were incubated in formamide at 37°C shaking for 24 hours before OD 620 was measured and quantified against a standard curve.
Metastasis colony formation assay

Micrometastases in the 4T1-Luc tumor model were quantified in the lungs and axillary lymph nodes by a 6-thioguanine selection assay as described previously (18). Briefly, lungs and lymph nodes were isolated at indicated times post tumor inoculation. Lungs were digested at 4°C for 75 minutes in 1 mg/mL Collagenase IV (Gibco) and 6 U/mL elastase (Worthington Biochemical), filtered through a 70 µm nylon cell strainer, and cultured in 10 cm dishes in selection media of Iscove's Modified Dulbecco's Medium supplemented with 10% FBS, 1X antibiotic-antimycotic (Life Technologies), and 60 µmol/L 6-thioguanine (Sigma). Lymph nodes were mechanically disrupted using a mortar and pestle (Kimble BioMassher) and cultured in 6-well plates in selection media. After 14 days in culture at 37°C and 5% CO₂ without disruption, colonies were fixed in methanol, developed using 0.03% (w/v) methylene blue (Sigma) in water, and colonies manually quantified.

Flow cytometry analysis

Antibodies to CD45 (30-F11), CD45.2 (104), CD3e (145-2C11), CD8α (53-67), CD8C (RM4-5), CD25 (PC61), NKp46 (29A1.4), NK1.1 (PK136), Ly6C (HLK14), Ly6G (1A8), CD11c (N418), CD11b (M1/70), I-A/1-E (MS5/14.5.2), Granzyme B (G111), IFNγ (XMGl2), PD-1 (29F.1A12), perforin (1S6009B), TNFα (MP6-XT22), IL2 (JE65–5H4), CD69 (H.12F3), IFNAR (MAR1–5A3), NKG2D (C6X), DNAM-1 (10E5), Fasl (MFL3), and mTRAIL (N2B2) were purchased from BioLegend. Antibodies to CD45 (30–F11) and K67 (B56) were purchased from BD Biosciences. FoxP3 (FJK-16s) antibody was purchased from Thermo Fisher Scientific. GFP tetramer (T-Select H-2L* MuLV gp70 Tetrramer–SPPSVYHYQFPE) was purchased from MBL. Tetramer staining was performed in buffer containing 50 mmol/L d-lasatiniib. Viability was assessed using LIVE/DEAD Fixable Aqua (Life Technologies) for tumor, lung, and spleen samples or using DAPI for tetramer staining of blood samples. FoxP3 staining and K67 staining were performed using the FoxP3 Transcription Factor Buffer Set (eBioscience).

Immune cell infiltrates of tumors, lungs, and spleens were analyzed as described previously (19); briefly, organs were harvested day 1, 3, or 6 after the start of treatment. Before tissue harvest, mice were injected 2 minutes prior to euthanasia retro-orbitally with an APC-Cy7 conjugated anti-CD45.2 antibody to label hematopoietic cells in the circulation but not the lung parenchyma. Tumors and lungs were cut into 1 to 2 mm size pieces and digested at 37°C for 30 minutes in the following enzyme solutions. 4T1-Luc: 10 mg/mL collagenase I (Gibco) and 40 U/mL DNase I (Roche); E0771 and lungs: 125 U/mL collage- nase IV (Gibco) and 40 U/mL DNase I in 1× HBSS. The digested tissues were then passed through a 70 µm nylon cell strainer. Cells were mechanically disrupted through 70 µm nylon cell strainers. All tissues were pelleted to remove digestion buffer, red blood cells were lysed in ACK lysis buffer (Gibco), and samples were washed in buffer containing 2% (w/v) BSA and 5 mmol/L EDTA (staining buffer). For intracellular cytokine/granule staining of tissue infiltrating cells, digested and lysed tissue samples were resuspended in staining buffer containing 1× brefeldin A (BioLegend) for at least 1 hour prior to staining. Cells were analyzed using a BD FACS LSR II, BD FACS LSR Fortessa, or BD FACS Canto flow cytometers. Data were analyzed in FlowJo.

Fluorescent CDN uptake

4T1-Luc tumor–bearing mice were treated intratumorally with a mixture of 30 µg 2′3′-c-di-AMP(S)₂, and 5 mmol sulfo-Cy5 labeled 2′3′-c-GAMP (Biolog) in 30 µL sulfo-Cy5 2′3′-c-GAMP was selected because of lack of commercially available labeled 2′3′-c-di-AMP(S)₂. Tumors, blood, and lungs were collected 2, 6, and 24 hours after injection and processed as described above. Cy5 signal was analyzed by flow cytometry using a BD FACS LSR Fortessa.

IFNβ reporter assay

A total of 5 × 10⁵ E0771 cells were inoculated into the fourth mammary fat pad of IFNβ reporter mice. Two weeks after tumor inoculation, mice received one dose of CDN (30 µg i.t.), Alb-IL2 (30 µg i.p.), and anti–PD-1 (200 µg i.p.). A total of 24 hours after treatment tumors and lungs were processed for flow cytometry according to the protocol above. CyFP signal was analyzed using a BD FACS LSR Fortessa.

Serum cytokine analysis

Serum samples were collected from 4T1-Luc tumor–bearing mice by retro-orbital bleed 6 and 24 hours after one dose of combination immunotherapy and frozen at –20°C until analysis. Samples were diluted 1:1 with Assay Buffer and assayed using the LEGENDplex mouse antiviral response panel (BioLegend) according to manufacturer’s instructions. The cytokometric bead array readout was performed using a BD FACS LSR Fortessa cytometer and analyzed using LEGENDplex v8.0 software.

NK-cell isolation and in vitro stimulation

For in vitro stimulation assays, NK cells were isolated from spleens of 7- to 10-week-old Balb/c mice using an EasySep Mouse NK cell Isolation Kit (STEMCELL) according to the manufacturer’s instructions. Cells were seeded at 50,000 NK cells per well in RPMI1640 with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1× minimum essential medium non-essential amino acids (Gibco), and 1 mmol/L sodium pyruvate (Gibco). Alb-IL2, IFNβ, and IFNg were added in complete medium as indicated. Cells were incubated at 37°C and 5% CO₂ and stained with antibodies for flow cytometry 24 hours after stimulation unless indicated otherwise.

Statistical analysis

Statistics were performed using Prism (GraphPad). Statistical methods were not used to determine sample size, but sample numbers were chosen on the basis of estimates from pilot studies and published results, such that appropriate statistical tests would yield statistically significant results. For survival studies, log-rank (Mantel–Cox) tests were used. For immune cell infiltration studies, NK functional markers analyzed at multiple time points, and serum cytokine analysis two-way ANOVA tests were used followed by Dunnett multiple comparisons test versus untreated controls. For IFNAR blockade lung analysis, two-way ANOVA tests were used followed by Tukey multiple comparisons test. Unpaired two-tailed Student t test was utilized for analysis of NK functional markers induced by CIP versus untreated controls at a single time point. Significance is indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. The n values and specific statistical methods are indicated in figure legends.

Results

Neoadjuvant CIP therapy promotes primary tumor regression and inhibits metastasis

We first explored combinations of cyclic dinucleotide (CDN) STING agonists, extended half-life Alb-IL2, and anti–PD-1 checkpoint blockade in a neoadjuvant model of triple-negative breast cancer.
Primary tumors were established by injection of 4T1-Luciferase cells into the mammary fat pad of female Balb/c mice. When tumors reached approximately 100 mm³ in volume (8 days after inoculation) animals were treated with three doses of CDN alone every 3 days, two doses of Alb-IL2 and anti–PD-1 given 6 days apart, or the combination of all three agents, followed by surgical resection of tumors 9 days after start of treatment (CIP therapy; Fig. 1A). Intratumoral CDN monotherapy or the systemic Alb-IL2 and anti–PD-1 combination led to moderate primary tumor growth delay compared with untreated controls, but the triple combination halted primary tumor progression (Fig. 1B). Following tumor resection, untreated control animals succumbed to metastases with a median survival of 38 days. Treatment with CDN alone or the dual combination of IP resulted in median survival times of 44.5 and 47 days and long-term survival in 20% and 10% of mice, respectively. In contrast, CIP treatment increased the long-term survival rate to 60% (Fig. 1C). This enhancement in efficacy required all three components, as monotherapy or pairs of these agents were significantly less effective (Supplementary Fig. S1A). The combination treatment was well tolerated, with mice receiving CIP experiencing transient weight loss that recovered prior to surgery (Supplementary Fig. S1B). In addition, liver enzymes AST and ALT were elevated briefly following CIP treatment but returned to baseline by 72 hours after treatment (Supplementary Fig. S1C and S1D). Clinical use of recombinant IL2 is hindered by dose-limiting toxicities including increased lung vasculature permeability mediated by innate immune cell–induced damage to endothelial cells (20, 21). To assess the risk of enhanced vascular leak syndrome when combining Alb-IL2 with anti–PD-1 and STING agonist treatment, we evaluated alterations in lung vessel permeability by Evans Blue dye uptake. Although the lungs of animals receiving CIP treatment exhibited an approximately 75% increased uptake of intravenous administered Evans blue compared with untreated or Alb-IL2–treated controls, this increased permeability was still only 52% of that induced by traditional

**Figure 1.**
Combination of neoadjuvant CDN STING agonist therapy with systemic Alb-IL2 and anti–PD-1 improves survival in an orthotopic breast tumor model. A–C, Balb/c mice (n = 10 animals/group) were inoculated with 4T1-Luc cells in the mammary fat pad and then treated with 30 μg CDN 2’3’-c-diAMP(PS)2 (i.tu.), 30 μg Alb-IL2 (i.p.), and 200 μg anti–PD-1 (i.p.) or subcombinations of these agents as indicated, followed by surgical resection of the tumor. Shown are the treatment schedules (A), mean tumor volume (B), and survival curves (C). D–H, Neoadjuvant therapy as in A, with treatment commencing 12 days after tumor inoculation. Shown are treatment schedules (D), mean tumor volume (E), survival curves (F), representative bioluminescence images (G), and luminescence quantification (H). Data compiled from two independent experiments. Data represent the mean ± SEM. **, P < 0.01; ****, P < 0.0001; versus untreated by log-rank (Mantel–Cox) test.
high-dose IL2 therapy (Supplementary Fig. S1E). Altogether, this treatment appeared to be safe and effective as a neoadjuvant therapy.

Analysis of micrometastases with a colony outgrowth assay on lung tissues at various time points after primary tumor inoculation pinpointed metastatic seeding of the draining axillary lymph node and lungs between 10 and 14 days after mammary fat pad injection (Supplementary Fig. S2A and S2B). To evaluate whether CIP could eliminate tumors in mice with definitively established micrometastases, we delayed the start of treatment until day 12 after tumor cell inoculation, when primary tumors were approximately 200 mm³ (Fig. 1D). In this setting, treatment with CDN or Alb-IL2/anti–PD-1 delayed primary tumor growth, but all animals succumbed to metastatic disease following primary tumor resection at a rate indistinguishable from untreated controls (Fig. 1E and F). The triple combination, however, resulted in regression of primary tumors, and following surgery, 30% of mice survived long term (Fig. 1E and F).

Postresection bioluminescence imaging revealed expansion of metastases distal to the primary tumor site as early as 1 week after resection in untreated, CDN monotherapy, and IP dual therapy groups (Fig. 1G). We also tested this treatment in C57Bl/6 mice, orthotopically implanting E0771 breast tumors. In this model, neoadjuvant CIP was similarly well tolerated and led to 100% survival following primary tumor resection, whereas only 23% of mice left untreated following resection survived longer than 100 days, with a median survival time of 53 days (Supplementary Fig. S3A–S3D).

Given the efficacy of CIP, we next examined whether neoadjuvant or adjuvant administration was more effective in the 4T1-Luc model. To initiate adjuvant therapy at a time point before an overwhelming metastatic burden was established, we compared neoadjuvant treatment beginning on day 6 when tumors were approximately 40 mm³ against treatment beginning 3 days after surgery (Fig. 2A). Neoadjuvant CIP resulted in 90% overall survival, whereas only 1 of 10 mice survived following adjuvant immunotherapy (Fig. 2B).

Figure 2. Maximal efficacy of CIP immunotherapy requires neoadjuvant treatment scheduling and surgical resection of the primary tumor. A, Timeline and components of treatment consisting of 30 μg CDN Z’3’-cdA(PS)2 (i.tu.), 30 μg Alb-IL2 (i.p.), and 200 μg anti–PD-1 (i.p.). Surgical removal of primary tumor was conducted on day 15. B, Survival curves for mice treated as in A. In B, n = 10 Balb/c mice per group, and data were compiled from two independent experiments. Mean tumor volume (C) and survival over time (D) for mice treated as in A without surgical resection of the primary tumor. In C and D, n = 9 Balb/c mice per group. Data in C represent mean ± SEM. *** P < 0.001; **** P < 0.0001; versus neoadj. log-rank (Mantel-Cox) test.
tumor. CIP therapy administered beginning on day 6 arrested primary tumor growth up to 30 days after inoculation, but most primary tumors progressed by day 40, leading to poor overall survival (Fig. 2C and D; Supplementary Fig. S4A). When treatment commenced 8 days after tumor inoculation, in the absence of surgical resection, both CI and CIP treatment yielded tumor clearance in about 40% of mice (Supplementary Fig. S4B–S4D). The greater efficacy of CIP over CI treatment in the setting of neoadjuvant therapy, but not in the absence of surgery, suggests the triple combination enhances immune control of residual metastases, but addition of checkpoint blockade is not important for control of the primary tumor. Altogether, treatment in the neoadjuvant setting with surgical intervention maximizes the therapeutic benefit of CIP combination therapy.

**CIP treatment induces changes in immune cell infiltration at the primary tumor and lungs**

As STING agonism, IL2, and checkpoint blockade have each been documented to engage both innate and adaptive immune cells, we profiled immune cell infiltration in primary tumors and the lungs as the predominant site of metastasis (Supplementary Fig. S5A; refs. 18, 22–24). Within primary 4T1 tumors, treatments containing Alb-II2 enhanced infiltration of CD8\(^+\) T cells (Fig. 3A). Following monotherapy treatment, CD4\(^+\) effector T cells and regulatory T cell (Treg) numbers declined from 3 to 6 days at the primary tumor, but CIP led to sustained numbers (Fig. 3B and C). Despite increases in Tregs within the primary tumor, Alb-II2 monotherapy drove increased CD8\(^+\)/Treg ratios compared with untreated controls, and CIP trended toward an increased ratio (Fig. 3D). Both CDN monotherapy and the triple combination treatment also elicited significantly increased primary tumor infiltration of CD3\(^+\)NKp46\(^+\) NK cells as early as 3 days following the first dose of treatment (Fig. 3E). Absolute NK-cell numbers in these two groups continued to rise within primary tumors, reaching a peak of around 10-fold over untreated controls 6 days after treatment. Cohorts receiving intratumoral CDN or CIP exhibited increased primary tumor neutrophil counts (Fig. 3F).

In the lungs of both 4T1-Luc and E0771 tumor-bearing mice, CD8\(^+\), CD4\(^+\), and Treg infiltration was elevated 3 days after commencement of treatment with IP. In both models, CIP similarly increased CD8\(^+\) and Treg infiltration in the lungs, but CD4\(^+\) effector cells were less abundant than post-IP treatment (Fig. 3G–I; Supplementary Fig. S6A–S6C). At 3 days after treatment, T-cell counts in the spleen were not significantly different from controls, but CD8\(^+\) T cells and Tregs showed a transient expansion in the blood at 3 days post CIP, followed by a contraction at day 6 (Supplementary Fig. S7A–S7F). High numbers of NK cells were present in the lungs of tumor-bearing mice irrespective of treatment condition, peaking at day 3 but remaining high 6 days after treatment (Fig. 3; Supplementary Fig. S6D). In contrast, there was no increase in total NK-cell count within the spleen or blood following immunotherapy administration (Supplementary Fig. S7G and S7H). Although neutrophils increased in primary tumors with time, they were depleted from the spleen, blood, and blood following CIP therapy (Fig. 3K; Supplementary Fig. S7i and S7j). Classical CD11b\(^+\)MHC\(^{low}\)Ly6G\(^+\) Ly6Chi inflammatory monocytes showed no treatment-induced changes in infiltration in the lungs, spleen, and blood (Supplementary Figs. SSB, and S7K and S7L); however, nonclassical patrolling monocytes (CD11b\(^+\)MHC\(^{low}\)Ly6G\(^+\)Ly6C\(^{low}\)) transiently infiltrated the lungs in CIP-treated mice (Supplementary Fig. S5C). Overall, CIP treatment altered both adaptive and innate leukocyte proportions within intratumorally injected and noninjected tissues.

**Long-term survival following CIP therapy is independent of T cells and dependent on NK cells**

To determine which immune cells govern therapeutic responses to CIP treatment, we performed cellular depletion studies in the 4T1 model (Supplementary Fig. S8A). Depletion of neutrophils, CD8\(^+\) T cells, or CD4\(^+\) T cells did not impact primary tumor progression nor survival during CIP treatment (Fig. 4A–C; Supplementary Fig. S8B–S8D). In contrast, an approximate 50% reduction in survival rate was observed when NK cells were depleted (Fig. 4D; Supplementary Fig. S8E).

To further examine the role of NK cells in the response to CIP, we treated 4T1 tumors in NOD.Cg-Prkdecid/LtJ (NOD SCID) mice, which lack functional B and T cells (25), and NOD.Cg-Prkdecd/J II2rg\(^{tm1Wjl}\)/SzJ (NSG) mice, which lack B and T cells as well as functional, mature NK cells (26). CIP therapy delayed primary tumor growth in NOD SCID animals, though to a lesser degree than Balb/c mice; tumor progression was even less impacted in NSG mice (Fig. 4E). Following surgical resection, no significant survival benefit was detected for CIP-treated NSG mice compared with untreated controls (Fig. 4F). In contrast, 78% of treated Balb/c mice and 56% of treated NOD SCID mice survived greater than 90 days, suggesting that NK cells play an important role in preventing metastatic disease (Fig. 4F).

Despite the maintenance of therapeutic response in the absence of CD8\(^+\) T cells, several lines of evidence suggested that CIP treatment induced an adaptive immune response. Analysis of peripheral blood 2 weeks after tumor resection revealed increased frequencies of CD8\(^+\) T cells specific for the endogenous retroviral antigen gp70 expressed by 4T1 cells (27) following CIP therapy (Supplementary Fig. S9A). Mice surviving greater than 90 days following CIP neoadjuvant therapy were rechallenged with 4T1-Luc tumor cells in the hind flank. Animals that had received neoadjuvant CDN monotherapy exhibited tumor outgrowth on rechallenge indistinguishable from naive mice, but animals that had received neoadjuvant CIP or subcombinations of the triple therapy exhibited significantly delayed secondary tumor growth (Supplementary Fig. S9B). Two weeks after rechallenge, animals previously treated with neoadjuvant CIP showed substantial expansion of gp70-specific T cells in the blood (Supplementary Fig. S9C). Depletion of CD8\(^+\) T cells during rechallenge resulted in a significant acceleration of rechallenge tumor growth in mice previously treated with CIP compared with controls (Supplementary Fig. S9D). During primary tumor challenge, activated (CD44\(^+\)IFN\(^\gamma\)^+\) CD8\(^+\) T cells expanded by percentage and number in the lungs (Supplementary Fig. S9E). Finally, expression of PD-1 and Tim-3 by lung CD44\(^+\) or CD44\(^+\)IFN\(^\gamma\)^+\) T cells was largely unchanged in treated versus untreated conditions (Supplementary Fig. S9F and S9G). Thus, although T cells do not play a role in protection from metastases in this model, antitumor T-cell responses are induced by CIP treatment.

**NK-cell activation is sustained by CIP therapy**

We next examined functional markers expressed by the lung-infiltrating NK-cell population following treatment. CIP therapy led to substantial increases in the fraction of NK cells expressing the cytotoxic granule components granzyme B and perforin; cytokines TNF\(\alpha\), IFN\(\gamma\), and IL2; early activation marker CD69; proliferation marker Ki67; and the exhaustion marker PD-1 (Fig. 5A). We next sought to examine the behavior of granzyme B and PD-1 over time and to dissect the impact of individual components of the combination therapy. CIP induced an increase in NK-cell granzyme B expression in the lungs significantly higher than that elicited by CDN or Alb-II2
Figure 3.
CIP immunotherapy alters adaptive and innate immune cell infiltration in primary tumors and lungs. A–K, Mice were inoculated with 5 × 10^5 4T1-Luc tumor cells in the mammary fat pad and treated with CIP or the individual agents when tumors reached 50 to 100 mm^3. Age-matched naive, non-tumor-bearing mice were used as controls. Tumors (A–F) and lungs (G–K) were isolated 1, 3, or 6 days after initiation of treatment, as indicated, and cells quantified by flow cytometry. Shown are tumor-infiltrating CD8^+ T cells (A), CD4^+ effector T (Teff) cells (B), Tregs (C), CD8/Treg ratio (D), NK cells (E), and neutrophils (F); lung-infiltrating CD8^+ T cells (G), CD4^+ T^eff cells (H), and Tregs (I) from 3 days after treatment; and lung-infiltrating NK cells (J) and neutrophils (K) from 1, 3, and 6 days after treatment. Data shown are mean ± SEM and are representative of at least two independent experiments (n = 4 naive or 5 tumor-bearing mice per group per time point). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 versus untreated at each time point by two-way ANOVA followed by Dunnett multiple comparisons test.
monotherapies at all time points (Fig. 5B). Granzyme production, measured by mean fluorescence intensity of the granzyme B+ population, was also highest in the triple combination group (Fig. 5B). Similar prolonged activation of NK cells was observed in lung-infiltrating NK cells of E0771 tumor-bearing mice (Supplementary Fig. S10A). CIP treatment also led to an increase in the fraction of NK cells expressing PD-1 and increased the level of PD-1 expression (Fig. 5C). CIP treatment or were untreated (Supplementary Fig. S11C). Expression of both FasL and membrane TRAIL was more apparent in NK cells of E0771 tumor-bearing mice, and CIP treatment elevated surface expression of both death ligands (Supplementary Fig. S11D). Altogether, NK cells showed upregulation of multiple effector pathways in the lungs following CIP treatment, including sustained expression of granzyme B.

**STING activation-driven type I IFN synergizes with IL2 to promote granzyme expression in NK cells**

CDNs have a very short half-life in vivo (28) and hence we expected that CDNs were unlikely to directly stimulate STING in NK cells outside of the primary tumor. To measure direct uptake of CDNs by NK cells, we treated 4T1 tumor-bearing mice with CIP in the presence of a fluorophore-conjugated CDN. At 6 hours

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**Figure 4.** Efficacy of CIP therapy is independent of T cells and neutrophils but dependent on NK cells. A–D, Balb/c mice bearing orthotopic 4T1-Luc tumors were treated as in Fig. 1A in the presence of depleting antibodies specific for the indicated surface markers or isotype controls. Shown is overall survival following depletion of neutrophils (A), CD8+ T cells (B), CD4+ T cells (C), or NK cells (D). n = 15 mice per group for neutrophil depletion; n = 5 mice per group for CD8 and CD4 depletion; n = 10 mice per group for NK depletion. Data shown are one representative of two independent experiments. Percent survival in E represent mean ± SEM, ****, P < 0.0001 by log-rank (Mantel–Cox) test. ns, not significant.
after treatment, a small proportion of CD45$^+$ cells in the primary tumor took up the labeled CDN. Among these leukocytes, a proportion of NK cells internalized the STING agonist (Fig. 6A). In the blood and lungs, no uptake of CDN by NK cells was detected (Fig. 6B and C). It therefore seemed unlikely that the intratumorally administered STING agonist is directly acting on lung-infiltrating NK cells. However, downstream of intratumoral STING activation is production of type I IFNs by host and/or tumor cells (8, 29). Utilizing IFNβ reporter mice expressing enhanced yellow fluorescent protein (eYFP) in the IFNβ locus, CIP treatment of E0771 tumor–bearing mice generated a larger fraction of tumor-resident CD45$^+$ cells expressing eYFP 24 hours after treatment in tumors compared with untreated controls (Fig. 6D). Similarly increased IFNβ production was not observed within the lungs, signifying that CDNs stimulate the majority of type I IFN cytokine secretion outside of the lung tissue (Fig. 6E). Examination of immune cells comprising the eYFP$^+$ population revealed neutrophils as the primary IFNβ producers within the tumor microenvironment and CD11c$^+$ DCs as IFNβ producers within the lungs (Fig. 6D and E). Apart from the tumor, elevated levels of type I IFNs and IFNγ were observed in the serum 6 and 24 hours after the first dose of CIP therapy (Fig. 6F), suggesting that circulating type I IFN synergizes with IL2 to induce the phenotypic changes observed in lung NK cells.

Figure 5.

NK cells are activated in the lungs in response to CIP immunotherapy. Balb/c mice were inoculated with $5 \times 10^5$ 4T1-Luc tumor cells in the mammary fat pad and treated with CIP or individual agents as in Fig. 1A when tumors reached 50 to 100 mm$^3$. Lungs were isolated 1, 3, or 6 days after initiation of treatment, as indicated, and NK-cell functional markers quantified by flow cytometry. A, NK-cell expression of granzyme B, perforin, TNFα, IFNγ, IL2, CD69, Ki67, and PD-1 3 days post-treatment initiation. Representative flow cytometry plots, percentage of positive cells, and mean fluorescence intensity (MFI) for NK-cell expression of granzyme B (B) and PD-1 (C). Shown are mean ± SEM and are representative of two independent experiments (n = 5 animals/group per time point). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ versus untreated at each time point by two-way ANOVA followed by Student t test (A) or Dunnett multiple comparisons test (B and C).
Direct CDN uptake by NK cells is minimal and transient, whereas systemic IFNs are elevated with treatment. **A-C**, Balb/c mice were inoculated with 4T1-Luc cells in the mammary fat pad and treated intratumorally with a mixture of 30 μg 2’3’ c-di-AM(PS)2 and 5 nmoles sulfo-Cy5 2’3’ c-GAMP in 30 μL and intraperitoneally with Alb-IL2 and anti-PD-1. Shown are representative Cy5 (APC) histograms (**A-C**, left) and fraction of cell types that are Cy5+ in the tumor (**A**), blood (**B**), and lungs (**C**). Data shown are mean ± SEM and are representative of one independent experiment (**A-C**). **D**, and **E**, IFNβ reporter mice were inoculated with E0771 cells in the mammary fat pad and treated with one dose of 30 μg 2’3’ c-di-AM(PS)2 i.tu., 30 μg Alb-IL2 i.p., and 200 μg anti-PD-1 i.p. 2 weeks after tumor inoculation. A total of 24 hours after treatment, tumors (**D**) and lungs (**E**) were assessed for eYFP signal within the CD45+ population. Fraction of CD45+ cells expressing eYFP (top), eYFP MFI of CD45+ (middle), and proportion of immune cell types within the eYFP+ population (bottom) are shown (right). Data represent mean ± SEM from one independent experiment (**D** and **E**, left) or orb y (**D** and **E**, right). **F**, Balb/c mice inoculated with 4T1-Luc cells were treated as in **Fig. 1A**. Shown are serum cytokine levels 6 and 24 hours after one dose of CIP or indicated individual agents (**F**). Data shown are mean ± SEM and are representative of two independent experiments (**n = 5 animals/group**). **P** < 0.05; **P** < 0.01; **P** < 0.001 by two-way ANOVA followed by Dunnett multiple comparisons test versus CD45+ (**A-C**) or versus untreated (**F**) or by two-tailed Student t test (**D** and **E**). ns, not significant.
Consistent with this idea, titrated combinations of Alb-IL2 with IFNα or IFNβ generated synergistic, dose-dependent increases in the fraction of granzyme B–producing NK cells in vitro, mimicking the functional enhancements seen in NK cells in vivo (Fig. 7A). Sustained expression of granzyme B after stimulation in vitro with IFNβ and Alb-IL2 was also replicated (Fig. 7B).

To understand the source of this synergy between type I IFN and IL2, we dissected how combined stimulation of NK cells in vitro and in vivo might contribute to the differences of NK cell responses.

Discussion

In this study, we report the use of intratumoral CDN STING agonist therapy combined with systemic extended half-life IL2 and anti–PD-1 checkpoint blockade as an effective means to potentiate long-term survival following neoadjuvant therapy in models of metastatic triple-negative breast cancer. STING agonist treatment is known to cause tumor vasculature collapse, resulting in an initial wave of hemorrhagic necrosis followed by rapid influx of innate immune cells, including neutrophils (13, 30). Such a vasculature damage–mediated response is consistent with our finding that early tumor control was unaffected by early lymphocyte depletion. Although CIP combination therapy controlled primary tumor growth on a short time scale, combining CIP with surgical resection greatly enhanced overall outcomes, with a majority of animals surviving long-term. Overall survival, unlike primary tumor growth, was dependent on host immunity. Notably, neoadjuvant administration of CIP was significantly superior to adjuvant treatment. Previous studies using the 4T1 model of metastatic breast cancer theorized that survival benefit in the neoadjuvant setting may be limited to therapies that invoke antitumor T-cell responses (31). Here, we found that NK cells, rather than T cells,
were critical for prevention of metastases, despite evidence of a tumor antigen–specific T-cell response. Inflammation resulting from wound healing induced by surgical intervention may inhibit T-cell control of preexisting metastases (32), and surgical stress may further cause NK-cell dysfunction through downregulation of NK-cell adhesion molecules CD62L and CD11b and decreased KLRG1 expression (33), potentially explaining the discrepancy observed between treatment in the neoadjuvant and adjuvant settings.

Clinically, NK cells have been implicated in metastasis immunosurveillance across multiple cancer types, with high NK-cell infiltration of tumors correlating with positive clinical outcomes (34). In patients with breast cancer, NK-cell genes NKP46, NKp30, NKG2D, CRTAM, DNAM-1, CD96, LFA-1, and CD1d—implicated in NK- and NKT-cell activation and adhesion—provide a predictive signature of recurrence-free survival (35). Meta-analysis of microarray data from human primary breast tumor samples finds that nearly all breast cancer subtypes express NKG2D and DNAM-1 ligands, though NK-cell inhibitory receptors become upregulated as cancer progresses (36, 37). The near ubiquity of these NK-activating ligands across breast tumors (38) in parallel with apparent dysfunction of NK cells, motivates the development of therapies that recruit NK cells to sites of metastasis and promote NK-cell activation as a treatment paradigm to clear metastases.

Precedent from preclinical studies has been established for the NK-cell–activating effects of STING agonists, IL2, and PD-1 checkpoint blockade individually. In the case of STING agonism, either endogenous tumor-derived 2′,3′-cGAMP or exogenous administration of STING agonists leads to NK-mediated clearance of CD8+ T-cell–resistant solid tumors lacking MHC I expression (14, 15). Chemotherapies featuring STING agonists are reliant, in part, on NK-cell activation (39). Finally, clearance of metastases following sustained release of peripheroperative CDNs was dependent on both NK- and T-cell responses (40). IL2 has long been documented as an essential signal for NK-cell maturation, proliferation, and survival (41). In the context of NK-cell antitumor immunity, IL2 and other common gamma chain cytokines can reinvigorate NK cells from anergic phenotypes (42). Finally, conflicting evidence exists surrounding the function of PD-1 as a putative exhaustion marker for NK cells (24, 43). Despite the uncertain role of PD-1 on NK cells, we observe a survival benefit when adding PD-1 checkpoint blockade antibody to CDN + Alb-IL2 (Supplementary Fig. S1A), likely due to modulation of both NK and T cells (44, 45). Beyond PD-1, blockade of alternative coinhibitory receptors merits further study to overcome surgery-induced NK- and T-cell dysfunction. For example, NK-specific checkpoint receptors, such as NKG2A, may be relevant to explore; disruption of this signaling pathway or others may prove more synergistic with CI than PD-1 blockade.

When combined, CIP immunotherapy agents induced prolonged expression of several NK-cell effector molecules, including granzyme B, perforin, TNFα, and to a lesser extent IFNγ, with an important role for CDN-induced type I IFN in this response (Supplementary Fig. S12F). In vitro, both type I IFNs and IL2 independently led to large proportions of NK cells expressing granzyme B; however, only the combination led to sustained granzyme production. In murine NK cells, granzyme B is regulated at the translational level, as resting NK cells have high levels of preexisting mRNA that can be further enhanced by cytokine stimulation (46). Following IFNα stimulation of splenocytes, Gzmb is also one of the most highly upregulated, STAT1–dependent genes differentiating wild type versus STAT1−/− mice (47, 48), genetically demonstrating the link between type I IFN signaling and granzyme B expression. Likewise, STAT5, downstream of IL2 and other common gamma chain–utilizing cytokines, is known to bind the Gzmb locus (49). Wiedemann and colleagues found in MCMV viral infection that Gzmb is one of the most highly downregulated genes after infection in STAT5-deficient NK cells, highlighting the importance of STAT5 in NK-cell cytotoxicity (50). NK cells deficient in CD25 also display impaired antiviral immunity (50). Further studies are needed to definitively determine the mechanisms mediating IFN/IL2 synergy in granzyme production.

Toxicity remains a key challenge in the clinical translation of many combination immunotherapies and is often poorly predicted by mouse models. However, early clinical trials of STING agonists combined with PD-1 blockade, as well as combinations of tumor-targeted IL2 and checkpoint blockade, have already proven to be safe, with few dose-limiting toxicities (44, 51). Combinations of the long half-life IL2 molecule Bempedgaldeulin with PD-1/PD-L1 blockade have also been demonstrated to be tolerable, induce T- and NK-cell expansion, and increase expression of genes associated with T- and NK-cell cytotoxicity (44). Given the striking therapeutic efficacy we demonstrate here with combined STING agonist/IL2/ checkpoint blockade treatment in multiple orthotopic models of metastatic breast cancer, exploration of this combination in the clinic appears warranted.

Authors' Disclosures

D.J. Irvine reports personal fees from Eliquis Therapeutics, Strand Therapeutics, Repertoire Immune Medicines, Ankyra Therapeutics, and Yenn Therapeutics outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions


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