Natural Killer T-cell Immunotherapy in Combination with Chemotherapy-Induced Immunogenic Cell Death Targets Metastatic Breast Cancer

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Abstract

Natural killer T (NKT) cells are glycolipid-reactive lymphocytes that promote cancer control. In previous studies, NKT-cell activation improved survival and antitumor immunity in a postsurgical mouse model of metastatic breast cancer. Herein, we investigated whether NKT-cell activation could be combined with chemotherapeutic agents to augment therapeutic outcomes. Gemcitabine and cyclophosphamide analogues enhanced the potential immunogenicity of 4T1 mammary carcinoma cells by increasing the expression of antigen-presenting molecules (MHC-I, MHC-II, and CD1d) and promoting exposure or release of immunogenic cell death markers (calreticulin, HMGB1, and ATP). In 4T1 primary tumor and postsurgical metastasis models, BALB/c mice were treated with cyclophosphamide or gemcitabine. NKT cells were then activated by transfer of dendritic cells loaded with the glycolipid antigen α-GalCer. Chemotherapeutic treatments did not impact NKT-cell activation but enhanced recruitment into primary tumors. Cyclophosphamide, gemcitabine, or α-GalCer–loaded dendritic cell monotherapies decreased tumor growth in the primary tumor model and reduced metastatic burden and prolonged survival in the metastasis model. Combining chemotherapeutics with NKT-cell activation therapy significantly enhanced survival, with surviving mice exhibiting attenuated tumor growth following a second tumor challenge. The frequency of myeloid-derived suppressor cells was reduced by gemcitabine, cyclophosphamide, or α-GalCer–loaded dendritic cell treatments; cyclophosphamide also reduced the frequency of regulatory T cells. Individual treatments increased immune cell activation, cytokine polarization, and cytotoxic responses, although these readouts were not enhanced further by combining therapies. These findings demonstrate that NKT-cell activation therapy can be combined with gemcitabine or cyclophosphamide to target tumor burden and enhance protection against tumor recurrence. Cancer Immunol Res; 5(12); 1086–97. © 2017 AACR.

Introduction

Invariant natural killer T (NKT) cells are a specialized population of T cells with restricted T-cell receptor (TCR) rearrangements, Vα14-Jα18 paired with Vβ8.2/7/2 in mice and Vα24-Jα18 paired with Vβ11 in humans, that recognize endogenous and pathogen-derived glycolipid antigens presented via the nonpolymorphic MHC-like molecule CD1d (1). NKT cells regulate innate and adaptive immune responses and contribute to tumor immnosurveillance (2), likely through detection of stress-induced glycolipids, tumor-associated glycolipid antigens, and/or inflammatory cytokines. Therapeutic NKT-cell activation provides protection from tumor development and progression in animal models (3–9) and induces lasting antitumor immunity (5, 7). In clinical studies, NKT-cell activation therapies stabilized disease in head and neck cancer patients (10, 11) and prolonged median survival time in lung cancer patients (12). Activated NKT cells can kill tumor cells directly (13–15) or indirectly by regulating the activation and function of T cells (5, 7–9, 16–18), NK cells (4, 5, 8, 9, 17), dendritic cells (DCs; refs. 3, 7, 18), and B cells (16, 17, 19). NKT-cell activation also inhibits tumor angiogenesis (6), eliminates tumor-infiltrating monocytes (20), and inhibits the activity of tumor-induced myeloid-derived suppressor cells (MDSCs; ref. 5).

Using the 4T1 mammary carcinoma model that mimics advanced metastatic breast cancer, we found that NKT-cell activation via a single transfer of glycolipid-loaded DCs significantly enhanced survival, providing durable cures in 40% to 50% of mice (5). As repeated treatment with glycolipid-loaded DCs or cotransfer of expanded NKT cells did not provide an additional survival benefit (5), we investigated in this study whether NKT-cell activation therapy could be combined with chemotherapy to enhance survival outcomes.
Although chemotherapeutic agents are generally selected for their cytotoxic effects, a subset of these drugs also promotes immune-mediated recognition of cancer cells (21–23). This recognition is facilitated by induction of immunogenic cell death (ICD), a process of programmed cell death that enhances presentation of tumor antigens (21, 22). Chemotherapy-induced ICD is characterized by cell surface exposure of the endoplasmic reticulum (ER) protein calreticulin (CALR; refs. 24, 25), secretion of ATP (26–28), and release of the chromatin-binding protein high-mobility group box (HMGB)-1 (28, 29). Collectively, these chemotherapy-induced changes in cell surface composition and release of soluble damage-associated molecular patterns (DAMPs) stimulate DCs to present tumor antigens and initiate antitumor immune responses.

Tumor cells treated with chemotherapeutics exhibit increased susceptibility to killing by NKT cells in vitro (15, 30). Similarly, NKT-cell activation in combination with either cisplatin (31) or 5-fluorouracil (32) exhibited promising results in murine models of mesothelioma and colorectal cancer metastasis, respectively. However, as neither of these agents induces ICD (22), the potential for combining ICD-inducing chemotherapies with NKT-cell activation remains undefined. The aim of this work was to examine the effects of combining NKT-cell activation therapy with ICD-inducing chemotherapy to target metastatic breast cancer.

Combining NKT-cell activation with cyclophosphamide or gemcitabine treatments reduced metastatic disease and enhanced survival over individual therapies. Mice surviving to the experimental endpoint following combined treatments (or NKT-cell activation alone) were protected from a second tumor challenge, consistent with long-lasting immune memory against tumor-derived antigens. Indeed, enhanced survival was associated with induction of robust antitumor responses by CD8⁺ T cells and NK cells. These results demonstrate that NKT-cell activation therapy can be combined with ICD-inducing chemotherapies to enhance control of metastatic breast cancer.

Materials and Methods

Mice

Female BALB/c mice were purchased from Charles River Laboratories and used at 8 to 12 weeks of age. Mice were maintained in the Carleton Animal Care Facility at Dalhousie University (Nova Scotia, Canada), and experimental procedures were approved by the University Committee on Laboratory Animals, following guidelines established by the Canadian Council on Animal Care.

Flow cytometry

The following antibodies were purchased from eBioscience or BioLegend (clone names are in brackets): purified CD16/32 (97); FITC-labeled TCRβ (H57-597), CD4 (RM4-5), CD11c (H13), MHC-I (H-2Db; 34-1-2S); PE-labeled CD1d (1B1), CD49b (DX5), CD8α (53-6.7), CD69 (H1.2F3), Foxp3 (FJK.16s), granzyme B (GZNB), Gr-1 (RB6-8C5), IFNγ (eBiol17BA); peridinin chlorophyll-Cychrome 5.5-labeled CD3 (145-2C11), CD54 (2D10) (40); allopurinol-cyanin-labeled CD11b (M1/70), CD11c (H13), CD8ε (53-6.7), CD25 (PC61), MHC-II (I-A/E; M5/114.15.2), and TCRβ (H57-597). CD1d tetramers loaded with the α-galactosylceramide (α-GalCer) analogue PBS57 were obtained from the NIH Tetramer Facility (Emory University, Atlanta, GA). Prior to staining, cell samples were preincubated with CD16/32 antibody to block nonspecific binding.

Cells were incubated at 4°C for 20 minutes with surface staining antibody panels, washed, and fixed in 2% paraformaldehyde (Thermo Fisher Scientific; AC416780250). For intracellular staining, fixed cells were incubated in permeabilization buffer (BioLegend; 421002) and stained with cytokine, FoxP3, or granzyme B antibodies. Analysis was performed using a FACS-Calibur cytometer with CellQuest Pro software (BD Biosciences). A FACSaria cell sorter with FACS Diva software (BD Biosciences) was used to sort cell populations (>95% purity) for in vitro cytotoxicity assays.

4T1 cell culture

4T1 mammary carcinoma cells were obtained from ATCC (CRL-2539) in 2005. No additional authentication was performed. Mycoplasma testing was performed every 3 to 6 months using the VenorGem Detection Kit (Sigma-Aldrich; MP0025). Aliquots of 4T1 cells were passaged 4 to 12 times in culture as described previously (5). Cells were harvested in the logarithmic growth phase for in vitro and in vivo experiments.

Cell viability assay

4T1 cells were cultured overnight in 96-well plates (5 × 10³/well) and then treated 48 hours with 0 to 1,000 ng/mL gemcitabine (Hospira; 00409-0182-01) or 0 to 10 μg/mL mafosfamide (active analogue of cyclophosphamide; Toronto Research Chemicals; M110300). Cell viability was determined via MTT assay (Life Technologies; M6494). The ability of chemotherapeutics to disrupt colony formation was also tested. 4T1 cells were plated at 2.5 × 10³ per well in 24-well plates and treated with gemcitabine or mafosfamide for 48 hours. Tumor colonies were fixed with methanol and stained with 0.03% methylene blue. Colonies were counted using an immunospot colony-counting program (Cellular Technology Limited).

Expression of MHC molecules

Surface expression of CD1d, MHC-I, and MHC-II on 4T1 cells was assessed by flow cytometry 48 hours after treatment with vehicle, gemcitabine, or mafosfamide.

ATP and HMGB1 release assays

 Supernatants were harvested from chemotherapy-treated 4T1 cells. Extracellular ATP levels were measured at 16 hours by luciferin-based ATP activity assay (Calbiochem; 119107-1KT) using a Fluoroskan Ascent FL plate reader (Thermo Fisher Scientific). HMGB1 levels were measured at 24 hours by ELISA (IBL International; ST51011) using an Epoch microplate spectrophotometer (BioTek).

Cell surface CALR translocation

To fluorescently mark the ER, 4T1 cells were stably transduced with a luminal ER marker mCherry-KDEL (33). Cells were cultured on coverslips overnight prior to 4-hour treatment with gemcitabine (10 ng/mL) or mafosfamide (5 μg/mL). Cells were fixed with 0.25% paraformaldehyde, washed in PBS and blocking buffer, and stained with a rabbit anti-CALR antibody (Abcam; ab2907) for 30 minutes. Reactivity was detected with an Alexa Flour 488-conjugated secondary antibody to rabbit IgG (Invitrogen; A-11008). To confirm intracellular CALR expression, untreated cells were fixed with 4% PFA and permeabilized with...
0.1% Triton X-100 (Bio-Rad; 1610407) prior to staining. Confocal microscopy (Zeiss LSM 510 Meta) and flow cytometry (FACS-Calibur) were used to analyze surface mobilization of CD11b.

**Western blot analysis**

Cells were lysed in ice-cold lysis buffer (components from Sigma-Aldrich): 20 mmol/L Tris-Cl pH 8, 300 mmol/L KCl, 10% glycerol, 0.25% Nonidet P-40, 0.5 mmol/L EDTA/EGTA, 50 mmol/L sodium fluoride, and 100 μmol/L sodium orthovanadate. Lysates were cleared by centrifugation (25 minutes, 15,000 × g, 4°C), and protein concentrations were determined by Bio-Rad protein assay (Bio-Rad; 500-0006). Equal quantities of soluble denatured proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad; 1620112). Membranes were washed and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated sheep anti-mouse (Sigma-Aldrich; A5906) or goat anti-rabbit (Sigma-Aldrich; AQ132P) secondary antibodies. Membranes were treated with ECL Western blotting substrate (Bio-Rad; 1705060) and exposed to X-ray film (Kodak; 201697), or detected using a Bio-Rad VersaDoc imaging system.

**Primary 4T1 tumor model**

4T1 cells were resuspended in saline and injected orthotopically (2 × 10^5 cells in 50 μL) into the fourth mammary fat pad of female BALB/c mice. On day 12, mice received a single intraperitoneal injection of 30 mg/kg gemcitabine or oral treatment with 20 mg/kg cyclophosphamide (Sigma-Aldrich; P9414-1G). Doses of gemcitabine and cyclophosphamide were based on previous studies (34, 35). Serum was harvested at 24 hours for analysis of HMGCR and ATP. Primary tumors were also harvested at 24 hours and dissociated into single-cell suspensions to measure CALR expression on 4T1 cells. Infiltration of CD45^- DCs (CD11c^-), NK cells (CD49b^-TCRβ^-), T cells (TCRβ^), and NKT cells (TCRβ^CD1d-tetramer^-) was assessed by flow cytometry.

To test the effects of single and combined therapies on primary tumor growth, mice received intraperitoneal injections of gemcitabine (30 mg/kg on days 12, 14, and 16) or cyclophosphamide in their drinking water (20 mg/kg/day equivalent from days 12–16). On day 17, NKT cells were activated by intravenous injection of 2 × 10^7 α-GalCer^-loaded DCs. To examine immune activation, serum IFNγ and IL4 were measured by ELISA (Bioscience; 88-7314-22 and 88-7044-88) at baseline, 2 and 24 hours after NKT-cell stimulation. To examine metastasis, lungs were harvested on day 28, dissociated through sterile wire mesh, and cultured in 24-well plates with 60 μmol/L 6-thioguanine (Sigma-Aldrich; A4660; ref. 5). After 7 days, plates were fixed with methanol and stained with 0.03% methylene blue. Tumor colonies were enumerated using immunospot colony-counting software.

**MDSC characterization**

Blood was obtained via submandibular venipuncture. 70 μL of blood was collected in 10 μL of heparin sulfate (10,000 U/mL; Sigma-Aldrich; H7640). To analyze MDSCs by flow cytometry, blood leukocytes were stained using Gr-1 and CD11b antibodies. To test the immunosuppressive activity of circulating MDSCs, a T-cell suppression assay was performed as described previously (5).

**Immune function assays**

To characterize whether treatments enhanced CD8^+ T-cell and/or NK-cell-mediated antitumor immunity, CD49b^-TCRβ^- NK cells and CD8^- T cells were sorted and cultured at a 1:1 ratio with Oregon Green^-labeled 4T1 target cells. After 18 hours, supernatants were collected for analysis of IFNγ levels, and cytotoxicity against 4T1 cells was determined as described previously (5).

**Immune phenotyping**

On day 28 after initial tumor cell inoculation, splenocytes were isolated by mechanical dispersion through wire mesh, followed by red blood cell lysis. The frequencies of CD4^- T cells, CD8^- T cells, and TCRβ^- CD4^- CD25^ FoxP3^- regulatory T cells (Treg) were examined, along with expression of the cell surface activation marker CD69. To monitor intracellular cytokine polarization, cells were restimulated for 4 hours with phorbol 12-myristate 13-acetate (Sigma-Aldrich; P8139) and ionomycin (Sigma-Aldrich; I21504-1G) and fixed with methanol and stained with 0.03% methylene blue. 4T1 target cells were fixed and stained for intracellular IL17A and IFNγ, or granzyme B.

**Statistical analysis**

Data are expressed as mean ± SEM. A two-tailed Mann–Whitney U test was used to compare between two groups. Comparisons between more than two groups were made via Kruskal–Wallis analysis with Dunn posttest. Statistical significance was set at P < 0.05. Survival data were analyzed by log-rank (Mantel–Cox) significance test with Bonferroni-corrected thresholds. Statistical computations were performed using GraphPad Instat 3.02 and GraphPad Prism 7.02.

**Results**

Effects of chemotherapeutics on 4T1 cell viability in vitro

The cytotoxic effect of gemcitabine and mafosfamide on 4T1 mammary carcinoma cells was investigated by MTT assay at 48 hours. Gemcitabine, a nucleoside analogue, caused a dose-dependent cytotoxic response with up to 75%
cytotoxicity at 1,000 ng/mL (Fig. 1A). Similarly, the alkylating agent mafosfamide also exhibited a dose-dependent cytotoxic effect on 4T1 cells (Fig. 1B). Both gemcitabine and mafosfamide caused concentration-dependent decreases in the number of 4T1 colonies in a clonogenic assay (Fig. 1C and D).

**Effect of chemotherapeutics on tumor immunogenicity**

We also investigated whether gemcitabine or mafosfamide could alter the expression of MHC molecules on tumor cells. MHC-I and MHC-II mediate presentation of peptide antigens to conventional T cells, whereas CD1d presents glycolipid antigens to NKT cells (1). Gemcitabine and mafosfamide treatments dose dependently increased the expression of MHC-I, MHC-II, and CD1d (Fig. 2A).

Some chemotherapeutics stimulate the immune system by inducing ICD, a mechanism of programmed cell death that increases immune recognition of tumor cells (21–23). To determine whether gemcitabine and mafosfamide induce ICD in 4T1 cells, we monitored the expression of the classical ICD markers CALR, ATP, and HMGB1 following treatment. During ER stress, CALR is mobilized to the plasma membrane (24, 25) and serves as an "eat me" signal for DCs, promoting the presentation of tumor-derived antigens to T cells (21). Cell surface localization of CALR was monitored on nonpermeabilized 4T1 cells by confocal microscopy and flow cytometry. After 4 hours of gemcitabine or mafosfamide treatment, CALR translocation to the cell surface was significantly increased (Fig. 2B and C). Consistent with induction of ER stress, we observed increased phosphorylation of PERK and Eif2α in treated cells (Supplementary Fig. S1A). These preapoptotic events precede apoptosis-associated caspase-3 cleavage, and Annexin V staining (Supplementary Fig. S1B and S1C; ref. 24). ICD also involves the secretion of ATP, which recruits DCs to the tumor site and activates the inflammaosome (26, 27), and release of HMGB1, which is recognized by Toll-like receptor (TLR)-4 to induce DC maturation (29). We observed significant increases in ATP and HMGB1 release from gemcitabine- and mafosfamide-treated cells in vitro (Fig. 2D and E). ATP and HMGB1 can be released through multiple mechanisms: autophagic cell death, necroptosis, pyroptosis, and necrosis (23, 26, 28). Consistent with activation of autophagy, we observed increased lipidation of LC3 and degradation of SQSTM1 (p62) in 4T1 cells treated with mafosfamide or gemcitabine (Supplementary Fig. S1D). We also observed phosphorylation of MLKL (Supplementary Fig. S1E), which is associated with necroptosis (28), and cleavage of caspase-1 and caspase-7 (Supplementary Fig. S1F), which are associated with pyroptosis (23). These data implicate multiple modalities of cell death in response to mafosfamide and gemcitabine treatments.

We also observed elevated surface expression of CALR in vivo in primary 4T1 tumors harvested from chemotherapy-treated mice (Fig. 2F). Although serum ATP levels were below the detection limits of our assay, we observed increased serum levels of HMGB1 (Fig. 2G). These results are consistent with gemcitabine and cyclophosphamide inducing ICD in 4T1 cells in vivo. Finally, tumor-bearing mice treated once with either cyclophosphamide or gemcitabine exhibited an increase in tumor-infiltrating CD11c+ DCs (Fig. 2H). With additional chemotherapy treatments over days 12 to 16, we also observed increased infiltration of NKT cells and T cells, but not NK cells, into primary 4T1 tumors (Fig. 2I–K).

**Enhanced tumor control by combining chemotherapy with NKT-cell activation**

We previously demonstrated that adoptive transfer of α-GalCer–loaded DCs into tumor-responded mice reduces metastatic tumor burden and increases survival (5). In this study, we aimed to establish whether NKT-cell activation therapy could be combined with ICD-inducing chemotherapeutics to enhance survival outcomes. In primary tumor and postsurgical breast cancer models (Fig. 3A), mice received either gemcitabine (days 12, 14, and 16) or cyclophosphamide (days 12–16) treatments prior to adoptive transfer of α-GalCer–loaded DCs (day 17). NKT-cell activation via transfer of α-GalCer–loaded DCs significantly increases serum cytokine levels (5). We did not observe reductions in serum IFNγ or IL4 levels when NKT cells were activated following gemcitabine or cyclophosphamide treatments (Fig. 3B), suggesting that chemotherapy treatments did not impede NKT-cell function. Cyclophosphamide and gemcitabine treatments also had no stimulatory effects on NKT cells in vivo (Supplementary Fig. S2), except for increasing NKT-cell infiltration into primary tumors (Fig. 2I). Treatment with

![Figure 1](https://www.aacrjournals.org/CancerImmunolRes/article-pdf/5/12/1089/3234699/cancerimmunolres-2017-0229 supplement.pdf)

*Figure 1.* Gemcitabine and mafosfamide reduce viability of 4T1 cells. Viability of 4T1 cells treated with gemcitabine (A, 0–1,000 ng/mL) or mafosfamide (B, 0–10 μg/mL) was assessed at 48 hours by MTT assay (n = 4/group over four independent experiments). *, P < 0.05 compared with vehicle. The effect of gemcitabine (C, 0–1,000 ng/mL) and mafosfamide (D, 0–10 μg/mL) on 4T1 cells was also assessed at 48 hours using a clonogenic assay. Colonies were fixed, stained with 0.03% methylene blue, and quantified using an automated immunospot colony-counting program (n = 4/group over two independent experiments). †, P < 0.05 compared with vehicle.
cyclophosphamide, gemcitabine, or α-GalCer–loaded DCs alone reduced primary tumor growth in nonresected mice (Fig. 3C). Similarly, metastatic burden of 4T1 cells in the lungs of tumor-resected mice was reduced by monotherapies (Fig. 3D). Protective responses were further enhanced when NKT-cell activation therapy was combined with gemcitabine or cyclophosphamide. Consistent with these results, enhanced survival was observed in our metastasis model when NKT-cell activation was combined with cyclophosphamide (Fig. 3E) or gemcitabine (Fig. 3F) treatments. Mice that survived to day 120 had no detectable tumor cells by clonogenic plating assay, suggesting they were free of metastatic disease.

Effect of monotherapies or combination therapy on antitumor immunity

To determine whether mice surviving the initial tumor challenge would have lasting immunologic memory against 4T1 tumors, we rechallenged mice by inoculating 4T1 cells in the contralateral mammary fat pad. Consistent with our previous observation (5), mice that received NKT-cell therapy exhibited...
reduced tumor growth and metastatic burden after rechallenge (Fig. 4A and B). Mice that received NKT-cell activation in combination with cyclophosphamide or gemcitabine exhibited attenuated tumor growth, similar to NKT-cell activation alone. Mice that had been treated with gemcitabine alone exhibited delayed tumor outgrowth but were unable to control the tumors (Fig. 4A). These mice also exhibited increased metastatic burden compared with mice that received combination treatments or NKT-cell activation alone (Fig. 4B).

Effects of therapy on suppressive immune cell populations
Accumulation of immunosuppressive MDSCs has been associated with cancer progression and poor therapeutic outcomes (36). We found that NKT-cell activation therapy decreased the frequency and suppressive function of CD11b⁺ Gr-1⁺ MDSCs in the 4T1 tumor model (5). To determine whether gemcitabine or cyclophosphamide in combination with NKT-cell immunotherapy would further impact MDSC levels, we examined the frequency of circulating CD11b⁺ Gr-1⁺ cells (Fig. 5A). Primary tumor resection transiently decreased the frequency of CD11b⁺ Gr-1⁺ cells (Fig. 5A). Accumulation of CD11b⁺ Gr-1⁺ cells was delayed in mice that received either gemcitabine or cyclophosphamide treatments alone (Fig. 5A). When combined with NKT-cell activation, neither cyclophosphamide nor gemcitabine cotreatment reduced the frequency of CD11b⁺ Gr-1⁺ cells below what was observed after NKT-cell activation alone. To investigate the immunosuppressive activity of CD11b⁺ Gr-1⁺ cells, we performed T-cell suppression assays using blood leukocytes isolated from naive and treated mice (Fig. 5B). Although blood cells from mice that received unloaded control DCs exhibited suppressive...
cancer tissue, all therapeutic interventions reduced the suppressive activity of MDSCs at day 35 after tumor inoculation, consistent with the reduced frequency of circulating CD11b+ Gr-1+ cells. Tregs have been shown to impede antitumor immunity (37). Consistent with published reports (37), chemotherapeutic treatment reduced the frequency of FoxP3+ Tregs in the spleen of tumor-resected mice (Fig. 5C), with or without NK-cell activation therapy. There was no effect of gemcitabine treatment, or NK-cell activation alone, on the frequency of Tregs.

T-cell activation following NKT-cell and chemotherapy treatments

Although chemotherapy can induce ICD of 4T1 cells, immune cells are also potentially sensitive to cytotoxic or cytostatic effects of chemotherapy. To better understand how chemotherapy alone or in combination with NKT-cell activation therapy influences the immune system, we characterized T-lymphocyte subsets in the spleen of tumor-resected mice. The total number of CD4+ and CD8+ T lymphocytes was not significantly altered by any of the treatments (Fig. 6), suggesting that the chemotherapeutic doses used were not toxic against T lymphocytes. The proportions of CD4+ and CD8+ T cells expressing the activation marker CD69 were increased in mice receiving gemcitabine, cyclophosphamide, NKT-cell activation, or combination therapies (Fig. 6), consistent with reported immunostimulatory activities of chemotherapeutics (27, 38–42) and NKT-cell activation (7). On their own, gemcitabine and cyclophosphamide treatments increased the frequency of IL17A+ CD8 T cells, but did not significantly increase the frequency of IFNγ+ T cells. IL17A- and IFNγ-expressing CD4+ and CD8+ T cells were increased by NKT-cell activation therapy, but frequencies were not enhanced further by combined therapy with gemcitabine or cyclophosphamide. Similarly, gemcitabine plus NKT-cell activation therapy increased granzyme B expression in T cells, but this was not significantly different from NKT-cell activation alone. Overall, additive or synergistic responses were not observed when NKT-cell activation therapy was combined with chemotherapy.

The effects of monotherapies and combination therapies on effector immune responses against 4T1 cells were investigated by assaying cytotoxic activity and IFNγ production by splenic NK cells and CD8+ T cells isolated from tumor-resected mice. NK cells and CD8+ T cells isolated from mice treated with NKT-cell–activating α-GalCer–loaded DCs alone, or in combination with gemcitabine or cyclophosphamide, mediated enhanced cytotoxic activity against 4T1 cells compared with cells isolated from mice treated with unloaded DCs (Fig. 7A and B) and released greater quantities of IFNγ in 4T1 cocultures (Fig. 7C and D). Gemcitabine or cyclophosphamide alone did not significantly increase cytotoxic responses (Fig. 7A and B). Cyclophosphamide treatment did not significantly increase IFNγ production from CD8 T cells or NK cells, but CD8+ T cells from gemcitabine-treated mice exhibited increased IFNγ release when cultured with 4T1 cells (Fig. 7C and D).

Discussion

Using a mouse model of breast cancer metastasis, we previously demonstrated that NKT-cell activation reduces metastatic burden, stimulates antitumor immunity, and improves survival (5). However, cures were only achieved in 40% to 50% of mice, even when NKT-cell activation therapy was repeated. This suggests that NKT-cell therapy needs to be combined with other therapies to elicit enhanced clinical outcomes. In this study, we demonstrated that NKT-cell activation therapy combined with gemcitabine or cyclophosphamide further enhanced protection against tumor burden and improved survival.

Chemotherapeutics are designed to kill cancer cells, but may do so by different mechanisms. Some chemotherapeutics promote immune responses against tumor cells by inducing ICD, a process that enhances uptake and presentation of tumor antigens by the immune system (21, 25–27, 29). This is in part due to the exposure or release of specific DAMPs, which recruit antigen-presenting cells, stimulate phagocytosis, and induce maturation. Although the list of chemotherapy-induced DAMPs continues to increase, the best characterized include CALR, ATP, and HMGB1 (21, 22). CALR exposure is dependent on the induction of a preapoptotic ER stress response and the anterograde translocation of CALR to the cell surface (24). Surface-exposed CALR binds to CD91 on macrophages and DCs, leading to the engulfment of dying and stressed cells, release of proinflammatory cytokines and priming of adaptive immunity (21, 22). ATP is a chemoattractant for antigen-presenting cells (21), and signaling via P2RX7...
receptors on DCs and macrophages leads to activation of the NLRP3/caspase-1 inflammasome pathway, with subsequent release of IL1\(\beta\) and IL18 (27). In particular, IL1\(\beta\) is required for the hierarchical priming of IL17\(^{+}\)γδ T cells and IFNγ\(^{+}\)CD8\(^{+}\) T cells (27, 38). HMGB1 released from dying cancer cells is a ligand for TLR-4 (29). HMGB1 binding to TLR-4 on DCs enhances processing of phagocytic cargo, facilitates antigen presentation, upregulates costimulatory molecules, and increases production of pro-IL1\(\beta\) (29). We observed that gemcitabine and mafosfamide induced multiple cell death pathways in 4T1 cells and increased the exposure or release of CALR, ATP, and HMGB1. As NKT-cell stimulation enhances NK-cell (4, 5, 13, 17) and T-cell (5, 7, 16–18) polarization and effector responses, we reasoned that coupling NKT-cell therapy with ICD-mediated presentation of tumor antigens would enhance antitumor immunity and survival.

Tumor cells can escape immunosurveillance via downregulation of classical MHC-I/II-mediated peptide antigen presentation to CD8\(^{+}\) and CD4\(^{+}\) T cells, or glycolipid antigen presentation to NKT cells via CD1d (14). Consequently, chemotherapeutics that restore antigen presentation by cancer cells could help reinstate tumor immunosurveillance. Consistent with reports of chemotherapy-induced upregulation of MHC expression (43), gemcitabine and mafosfamide increased expression of MHC-I and MHC-II on 4T1 cells. We also observed increased expression of CD1d, which is potentially important as NKT cell–mediated cytotoxicity typically requires CD1d recognition for effective killing (13–15).

Figure 5. Effect of therapy on immunosuppressive MDSCs and Tregs in the 4T1 model. A, Frequency of circulating Gr-1\(^{+}\)CD11b\(^{+}\) cells in mice treated with unloaded DCs, α-GaCer–loaded DCs, cyclophosphamide (CPX), gemcitabine (GEM), or combined therapies. Treatment groups were further separated to show mice that responded completely to therapy and mice that relapsed (\(n = 7–10\)/treatment group over three independent experiments). B, Proliferation of naive Oregon green-labeled responder T cells was examined by dye dilution assay following stimulation with anti-CD3/CD28 coated beads. Responder cells were incubated in the presence of peripheral blood leukocytes isolated from resected mice treated with unloaded DCs, α-GaCer–loaded DCs, CPX, GEM, or combination therapies. Histograms are representative, while the values indicate mean frequency of proliferating T cells ± SEM (\(n = 5–6\)/group over three independent experiments). *, \(P < 0.05\) compared with unloaded DCs. C, The frequency of TCR\(\beta\)^{+} CD4\(^{+}\) CD25\(^{+}\) Foxp3\(^{+}\) Tregs was measured in the spleen by flow cytometry (\(n = 7–8\)/group over three independent experiments). *, \(P < 0.05\) compared with treatment with unloaded DCs (white bar).
Consistent with previous reports, we observed increased survival in 4T1-challenged mice receiving cyclophosphamide (44), gemcitabine (45), or glycolipid-loaded DC treatment (5). Chen and colleagues (44) reported a survival rate of approximately 10% in 4T1 tumor-bearing mice receiving cyclophosphamide treatment; however, their study used a 4- to 8-fold higher dose of cyclophosphamide delivered intraperitoneally. In our study, we used a dose and administration route for cyclophosphamide that was compatible with immunotherapy in a vaccine study (35). Although cyclophosphamide prolonged survival, none of our mice survived to the experimental endpoint with cyclophosphamide treatment alone. Others have shown gemcitabine treatment in a postsurgical 4T1 metastasis model yields approximately 28% survival (45), consistent with our observations. Although these drugs induced features of ICD and reduced accumulation of immunosuppressive cells, these effects on their own may not be sufficient to overcome immune barriers to induce effective long-term immunity, as evidenced by poor control of secondary 4T1 challenges in gemcitabine-treated mice. Additional immune stimulation induced by NKT-cell activation may help overcome barriers to the establishment of long-term memory.

Few studies have examined NKT-cell activation in combination with chemotherapy. In a mouse model of mesothelioma, α-GaLCer treatment improved cisplatin-mediated protection (31). Similarly, α-GaLCer plus 5-fluorouracil enhanced protection from MC38 colorectal cancer metastasis over α-GaLCer or 5-fluorouracil treatments alone (32). We found that combining NKT-cell activation with cyclophosphamide or gemcitabine significantly increased survival. Chemotherapy treatment alone did not induce robust CD8+ T-cell or NK cell–mediated cytotoxicity against 4T1 cells, despite upregulating the frequency of activated and cytokine-polarized T cells. It is possible that these cells were not specific for tumor antigens, as chemotherapy alone did not induce effective protection against tumor rechallenge. In contrast, NKT-cell activation therapy alone or in combination with chemotherapy significantly increased cytotoxic activity and IFNγ production by NK and CD8+ T cells and mediated resistance to tumor rechallenge. Although the mechanisms by which NKT-cell activation stimulates NK and CD8+ T cells are not entirely elucidated, NKT cells release cytokines, such as IL2 and IFNγ, that support increased NK and CD8+ T-cell proliferation and effector functions (13, 17). Activated NKT cells also stimulate DCs to enhance antigen cross-priming (18, 46), upregulate costimulatory molecules (3, 7, 18), and attract naïve CD8+ T cells (46). Consistent with these observations, depletion of either NK cells or CD8+ T cells attenuates the anticancer effects of NKT-cell activation (8, 9).

NKT-cell activation in cancer patients has been associated with increased IFNγ production and stabilization of disease (47). Like mice, adoptive transfer of glycolipid-loaded DCs in humans generates better responses than free glycolipid treatments, and increased survival time in a phase I/II trial involving lung cancer patients (12). Some clinical trials have observed extra benefit from combining autologous NKT-cell transfers with DC transfers (10, 11). In contrast, we did not observe benefits from additional NKT cells in our 4T1 metastasis model (5). However, mice have more
NKT cells than humans (1) and may not benefit from the additional boost.

The numbers of circulating MDSCs correlate with disease progression in preclinical (5, 45) and clinical studies (36). MDSCs are of particular interest because they can inhibit antigen presentation and T-cell responses (36). We have previously reported that circulating MDSCs could be used as a prognostic factor in NKT cell–based therapy, and that NKT-cell activation could overcome MDSC-induced immunosuppression (5). Cytotoxic compounds have been found to deplete MDSCs through mechanisms that are incompletely understood. Sinha and colleagues (45) demonstrated that gemcitabine-induced reductions in MDSCs resulted in restoration of IL12 production and decreased IL10 levels in the 4T1 model, reversing detrimental Th2 immune polarization. In contrast to gemcitabine, cyclophosphamide has been reported to drive expansion of MDSCs at doses >100 mg/kg delivered intraperitoneally (48, 49). This is 5-fold higher than the dose used in our work; furthermore, we delivered cyclophosphamide perorally, which is likely to decrease bioavailability. In our studies, cyclophosphamide transiently suppressed the accumulation of MDSCs after resection. However, it is possible that this was related to reduced tumor-induced myeloablation resulting from increased tumor burden, rather than immune stimulation, as all mice in this group ultimately failed to control tumor growth and succumbed to metastatic disease.

Cyclophosphamide has been shown to indirectly induce IFNγ and IL12A-expressing CD4+ T cells by disrupting the integrity of the gut mucosa, and allowing Gram-positive bacterial colonization of secondary lymphoid organs (39). In addition, cyclophosphamide transiently decreases the frequency of immunosuppressive Tregs and enhances T-cell priming (42). In our study, cyclophosphamide increased the frequency of activated T cells and decreased the frequency of Tregs, but failed to induce significant Th1 and Th17 polarization. This could be due to the relatively low dose of cyclophosphamide used in our experiments (39).

Gemcitabine decreases the frequency and/or function of MDSCs (45) and B cells (50), while sparing T cells (50). Indeed, gemcitabine has been reported to enhance the frequency of IFNγ+ CD4+ and CD8+ T cells in a murine model of oral cancer (41). We observed an increase in numbers of activated CD4+ and CD8+ T cells in gemcitabine-treated tumor-bearing mice; however, using low doses of gemcitabine, the increase in IFNγ+ CD4+ and CD8+ T cells did not reach statistical significance. Although combining NKT-cell activation with low-dose chemotherapy resulted in improved survival, we did not observe additive effects on expression of cytokines or cytotoxic molecules in T cells. It is possible that the ICD-inducing chemotherapies increased survival by targeting immune responses to the metastatic tumor sites more efficiently. Consistent with this, we observed increased infiltration of T cells and NKT cells into primary unresected 4T1 tumors treated with gemcitabine or cyclophosphamide. NKT-cell infiltration into tumors has been linked to better prognosis in neuroblastoma and colorectal carcinomas (2), and tumor regression in head and neck cancers (10).

In conclusion, we show that NKT-cell activation therapy can be safely and effectively combined with ICD-inducing chemotherapy to target metastatic breast cancer. The combination therapy was associated with induction of antitumor responses that protected surviving mice against tumor rechallenge. Our findings provide preclinical evidence supporting the development of therapeutic NKT cell–based chemo-immunotherapy to target metastatic breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Gebremeskel, B. Johnston

Study supervision: B. Johnston

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