Loss of Host Type-I IFN Signaling Accelerates Metastasis and Impairs NK-cell Antitumor Function in Multiple Models of Breast Cancer

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Abstract

Metastatic progression is the major cause of breast cancer-related mortality. By examining multiple syngeneic preclinical breast cancer models in mice lacking a functional type-I interferon receptor (Ifnar1−/− mice), we show that host-derived type-I interferon (IFN) signaling is a critical determinant of metastatic spread that is independent of primary tumor growth. In particular, we show that bone metastasis can be accelerated in Balb/c Ifnar1−/− mice bearing either 4T1 or 66cl4 orthotopic tumors and, for the first time, present data showing the development of bone metastasis in the C57Bl/6 spontaneous MMTV-PyMT-driven model of tumorigenesis. Further exploration of these results revealed that endogenous type-I IFN signaling to the host hematopoietic system is a key determinant of metastasis-free survival and critical to the responsiveness of the circulating natural killer (NK)–cell population. We find that in vivo–stimulated NK cells derived from wild-type, but not Ifnar1−/−, mice can eliminate the 4T1 and 66cl4 breast tumor lines with varying kinetics in vitro. Together, this study indicates that the dysregulated immunity resulting from a loss of host type-I IFN signaling is sufficient to drive metastasis, and provides a rationale for targeting the endogenous type-I IFN pathway as an antimetastatic strategy.

Introduction

Metastasis is the deadliest result of malignant disease. Standard clinical practice to avoid this outcome focuses on primary tumor treatment using chemotherapy, radiotherapy, and/or surgical resection. However, despite the obvious benefits of these interventions, it has been reported that tumor cell dissemination can occur prior to primary tumor diagnosis (1). It is therefore important to understand the processes that dictate the survival and outgrowth of these disseminated cells to guide the development of novel antimetastatic therapies. Work on the later stages of the metastatic cascade has uncovered numerous mechanisms of disseminated tumor cell survival and outgrowth, such as resisting apoptosis following detachment from the extracellular matrix (2), de-novo angiogenesis (3), and growth factor signaling pathways that promote the proliferation of disseminated cells (4). Many of these mechanisms appear unique to a specific metastatic site, supporting the well-established idea that the organotropism of certain cancers relies upon a favorable interaction between the tumor cell and target microenvironment (5).

Recently, the antitumor immune response has emerged as a key factor in determining metastatic spread. Tumor cells can use multiple immune-evasive tactics to survive at various metastatic sites (6), including our recent findings that show a requirement for breast tumor cells to suppress their innate type-I IFN pathway to colonize the bone (7). The type-I IFN family was originally characterized as a group of soluble factors produced by almost all cells in the body that were able to confer an antiviral state (8, 9). Our knowledge of these cytokines has since broadened to include their ability to directly inhibit the proliferation of disseminated cells (10, 11). Although IFN therapies have had mixed success in the clinic as direct tumoricidal agents, their involvement in tumor immune surveillance is currently the focus of much research. Of particular note, experiments with mice lacking a functional type-I IFN receptor (Ifnar1−/− mice) have demonstrated that endogenous IFN signaling to the host hematopoietic system is critical for mounting an immunologic response against chemically induced sarcomas (12). However, less is known about the role that type-I IFNs play during the antimetastatic immune response, especially in immune-competent models of breast cancer.
In line with our previous work (7), we hypothesized that type-I IFN signaling was a critical regulator of breast cancer progression, then blocking such signaling should be sufficient to drive metastasis irrespective of an impact on primary tumor growth. To address this, we studied the full course of disease in a model of spontaneous breast tumorigenesis (MMTV-PyMT) as well as in two orthotopic models (4T1 & 66cl4) of breast cancer metastasis in wild-type (WT) and Ifnar1−/− mice. We show model-specific differences in primary tumor growth and lung metastasis and uncover a common theme of accelerated bone metastasis in Ifnar1−/− mice—a critical site of tumor cell dormancy and relapse in breast cancer patients (13). In addition, we demonstrate that these differences in metastatic spread may be partly attributable to the functionality of the natural killer (NK) cell component of the host’s innate lymphoid population (14). Finally, this work shows a critical role for type-I IFN signaling in promoting the antimetastatic response and highlights the importance of examining the full course of disease when investigating tumor immunity.

**Materials and Methods**

**Mouse models and cell lines**

All animal procedures were conducted in accordance with the Australian National Health and Medical Research regulations on the use and care of experimental animals and approved by the La Trobe Animal Experimentation Ethics Committee. C57Bl/6 MMTV-PyMT mice (15) and C57Bl/6 Ifnar1−/− mice (16, 17) were used to generate C57Bl/6 PyMT-Ifnar1−/− and PyMT-Ifnar1 WT mice. BALB/c Ifnar1−/− mice have been described before (17).

The 4T1 and 66cl4 tumor cell lines were sourced directly from Fred Miller who derived the cell lines from a spontaneous mammary tumor that arose in a Balb/c mouse (18). The highly bone-metastatic 4T1.2 subclone of the 4T1 line was derived in, and sourced from, Professor Anderson’s laboratory (19). All cells were engineered to express the mCherry and/or Luciferase (Luc2) reporter genes through retroviral transduction Murine stem cell virus vector. The use of the Yac-1 murine leukemia cell line as a robust NK-cell target has been well established (20). All cell lines were cultured in a−MEM (5% FBS) except for the Yac-1 line that was grown in DMEM (10% FBS, non-essential amino acids, sodium pyruvate, l-glutamine; Gibco reagents). All adherent cells were passaged using EDTA (0.01% w/v in PBS) and were cultured for no longer than 4 weeks.

**Orthotopic tumor cell inoculation, tumor measurement, and ex-vivo imaging**

Single-cell suspensions of 1 × 10^5 4T1, 4T1.2, or 66cl4 tumor cells in PBS were injected into the 4th mammary gland of 8- to 12-week-old female BALB/c mice. Primary tumor size was measured 3 times per week using electronic calipers, and tumor volume (mm^3) was calculated as L (mm) × W (mm)^2/2. If indicated, primary tumors were resected when the tumor volume reached 500 mm^3 or in the case of some 66cl4 experiments, at 21 days after tumor cell inoculation. Mice were sacrificed upon signs of metastatic distress or when the cumulative primary tumor volume reached 1,500 mm^3. The extent of metastasis in particular organs was assessed using quantitative real-time PCR (qPCR) or visualized by histology as described below. In addition, some organs were rapidly excised from euthanized animals and subjected to ex vivo imaging using an IVIS Lumina XR-III (Caliper Life Sciences). Spectrally unmixed mCherry fluorescence or bioluminescent intensity 10 minutes after i.p. injection of 1.5 mg D-Luciferin (Gold Biotechnology) was normalized between all images in a group using Living Image 4.4 software (Caliper Life Sciences).

**NK-cell transfer and metastasis assay**

Ifnar1−/− hosts (5–6 per group) bearing orthotopic 4T1 tumors were i.v. infused with 300,000 (in 200 μL PBS−/−) immunomagnetically purified NK cells (as described in NK isolation methods below) from pooled splenocytes from three poly(I(C))-activated (250 μg i.p., 48 hours prior to isolation) WT or Ifnar1−/− donors on days 2, 7, 17, and 25 after tumor cell inoculation. Primary tumors were resected on day 15, and mice were harvested 31 days after tumor cell inoculation with organs collected for metastatic burden analysis via qPCR as described below.

**Quantification of metastatic burden**

Duplex qPCR was used to quantify metastatic burden (as previously described by Eckhardt and colleagues; ref. 21) by comparing the ratio of mCherry (present in tumor cells) and vimentin (NC_000068, present in all cells) sequences in genomic DNA preparations from homogenized and proteinase-K (100 μg/ml, 70663; Merck) digested lungs, spines, and femurs. Taqman reagents and StepOne Plus instrument were from Applied Biosystems (Life Technologies), and primers were as follows: mCherry fwd: 5’-GACGACCTCTAACAGCGCAGAGG-3’, rev: 5’-AGGTGATG TCACAATGGATGTTGAG-3’, hybridization probe: 5’FAM-CAGCTG CCCGCGGCTACA-3’TAMRA and vimentin fwd: 5’-AGCTGC CATCCTACGGACACATTTG-3’, rev: 5’-CGGAAGTGACGAGCG CACCTC-3’, hybridization probe: 5’VIC-CTCCATGCTTGCATGCTC CATCCTGCAGG-3’TAMRA. Gene expression/metastatic burden in the spontaneous MMTV-PyMT model required the analysis of PyMT mRNA expression levels in frozen, crushed tissues. RNA was isolated from tissues using Qiagen RNeasy kits (Qiagen), and 1 μg of RNA was reverse transcribed using the Moloney murine leukemia virus reverse-transcriptase enzyme (MoMuLV-RT; Promega) and anchored oligo-(dT) primers. PyMT cDNA abundance was quantified relative to the Rps27a housekeeping gene using the previously mentioned Taqman reagents and instrument with the following primers: PyMT fwd: 5’-CCACACATACGCCCAGCAT-3’, rev: 5’-GGCTCTGTTGCCTTCATGCTA-3’, hybridization probe: 5’FAM-CAGCTGACACGAGCAT-3’TAMRA. Rps27a was measured using a commercial gene expression assay (Life Technologies; cat. # Mm01180369_g1). Gene expression/metastatic burden (arbitrary units) was based on the quantification cycle (Cq) for PyMT relative to Rps27a or mCherry relative to vimentin and displayed as relative tumor burden (RTB) using the following equation:

\[
RTB = 10,000 / (2^{ΔCq})
\]

where \(ΔCq = Cq\) (target gene) − \(Cq\) (control).

**Histology and immunohistochemistry**

Prior to paraffin embedding and sectioning, all tissues were fixed in 10% neutral buffered formalin for 24 hours. Bone was decalcified in 20% EDTA (pH 8) for 2 weeks at room temperature after fixation. Cellular morphology was visualized by hematoxylin and eosin (H&E) staining. IHC detection of epithelial cells was used to confirm the presence of bone metastases with a previously described protocol (7). Briefly, tissues were subjected to heat-induced epitope retrieval (125 °C for 3 minutes under pressure) in citrate buffer (10 mmol/L sodium citrate, pH 6) before incubating with a pan anti-cytokeratin antibody (5 μg/mL,
Membranes were gently agitated at 4°C overnight in a 5% skim-milk solution containing antibodies against murine Irf7 (AbD Serotec; AHP1180; 1 μg/mL) or GAPDH (Cell Signalling; #8884; 1:5,000 dilution). Primary antibody binding was detected using an anti-rabbit horseradish peroxidase (HRP)–conjugated secondary antibody (raised in donkey) and visualized using ECL substrate (Amersham ECL Prime; GE Healthcare) and a digital image capture system (Syngene; In Vitro Technologies). Measurement of IFNα secretion was performed by plating 25,000 cells of interest in triplicate wells of a 96-well plate, which were allowed to adhere overnight before transfecting (Lipofectamine 2000; Invitrogen) with 10 μg/mL poly(I:C) for 6 hours. Transfection media were then carefully aspirated and replaced with 50 μL of complete growth media and allowed to incubate for 16 to 18 hours at 37°C/5% CO₂. Standard ELISA protocols were then used to detect IFNα secretion; briefly, 35 μL of cell-free supernatant was then transferred to immunoassay plates coated with mIFNa capture antibody (1:500; 22100-1/PBL assay sciences), incubated for 2 hours at room temperature before sequential incubations with mIFNa anti-serum (1:500; 32100-1; PBL assay sciences), anti-rabbit-HRP tertiary antibody (1:500), tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) for 30 minutes at room temperature before quenching the reaction and measuring the OD at 450 nm with λ correction at 570 nm.

Flow cytometric analysis
Analysis of blood immune cell populations was completed by flow cytometry using an LSR-II and FACSCanto II (BD Biosciences), and data were analyzed using Flowjo software (Treestar). Blood from the lateral tail-vein was used to profile the circulating immune populations after red blood cell lysis (155 mmol/L NH₄Cl, 10 mM MgCl₂, 5 mg/mL EDTA, pH 7.3) and staining with the following antibodies: CD11b-FITC (M1/70), CD8a-PerCP-Cy5.5 (53-67), CD4-APC (RM4-5), TCRγδ-FITC (H57-597), NKp46-BV421 (29AL1.4), Ly6C-GV421 (1A8), Ly6C-APC (HK1.2), Cdh12-PE (H1.2F3; all from BD Biosciences). Intracellular IFNγ accumulation was analyzed using a specially designed kit (554715; BD Biosciences) according to the manufacturer’s instructions after cells were stimulated with 20 ng/mL PMA and 1 μg/mL ionomycin for 4 hours in the presence of Golgi stop. For the analysis of NK-cell ligands and the Ifnar1 receptor present on the tumor cell surface, the following antibodies were used: H2D(d)-PE (34-5-8S), H2K(d)-FITC (SF1-1.1) (BD Biosciences), CD155-PE (TX56), Ifnar1-PE (MAR1-5A3) (Biolegend), and MULT1-PE (237104), pan-Rae-1-PE (186107), and H60-APC (205326) (R&D Systems).

NK-cell isolation and cytotoxicity assays
Standard 4-hour cytotoxicity assays were completed as described elsewhere (22). Briefly, splenic NK cells from naïve or poly(I:C) activated (250 μg i.p. 48 hours prior to isolation) WT, Ifnar1−/−, or animals injected with an Ifnar1-blocking antibody (MAR1-5A3; 200 μg i.p., 72, 48, and 24 hours prior to harvest) were immunomagnetically enriched (NK-cell enrichment kit; #19755; Stem Cell Technologies) according to the manufacturer’s specifications and suspended in complete NK-cell medium (phenol-red free RPMI 1640 containing: 10% FBS, β-Me, non-essential amino acids, l-glutamine, sodium pyruvate, all from Gibco). The indicated target populations were labeled with 15 μmol/L calcein-AM (C1413; Life Technologies) and Annexin-V FITC (556419; BD Biosciences) according to the manufacturer’s specifications. Samples were analyzed using a FACS ARIA III cell sorter (BD Biosciences) equipped with a 561-nm laser to optimally resolve the mCherry-positive tumor cells and ensure that the resulting profiles did not represent apoptotic leukocytes. Proliferation assays were performed in the presence of 1,000 IU/mL mIFNα or β (produced in-house) as previously described (21) using sulforhodamine-B binding assays.

Bone marrow reconstitution
Female BALB/c mice (6–8 weeks old) received two doses of 5.5 Gy irradiation 3.5 hours apart before an intravenous injection of 2 × 10⁶ bone marrow cells isolated from 6- to 8-week-old BALB/c WT or Ifnar1−/− mice. Six weeks later, reconstitution was confirmed by flow cytometric analysis (FACS) of Ifnar1 expression on blood cells (Ifnar1 antibody clone: MAR1-5A3). Ten weeks after reconstitution, recipients were inoculated with 4T1.2 tumor cells as described above. Subsequent to tumor resection, mice were monitored daily until each individual mouse showed signs of distress due to metastatic burden.

Statistical analysis
Differences in primary tumor size and immune populations were assessed with Student’s t tests, and metastasis assays (non-normally distributed data) were analyzed with Mann–Whitney U tests. Mantel–Cox log-rank tests were used to evaluate differences in metastasis-free survival. ELISA data were interpolated from a 4-parameter logistic regression of a standard curve using a free online program available at ElisaAnalysis.com (Leading Technology Group). GraphPad Prism software (GraphPad) was used for all analyses. P values: *, P ≤ 0.05; **, P < 0.005; ***, P < 0.0005.

Results and Discussion
Loss of type-I IFN signaling accelerates bone metastasis independent of primary tumor growth
We initially set out to determine if type-I IFN signaling could affect tumor development and metastasis in a model of breast
cancer with spontaneous tumor initiation. The tumors that develop in the viral oncogene-driven MMTV-PyMT model are thought to faithfully recapitulate the human disease through the stages of early hyperplasia, carcinoma in situ, invasive cancer, and sporadic pulmonary metastasis (23, 24). Importantly, this model allows the assessment of other factors on tumor initiation, unlike our previous analysis using aggressive transplantable models where type-I IFN signaling did not affect tumor growth, contrary to other studies with sarcoma models (12). Nevertheless, in line with our previous study (7), crossing these mice to hosts lacking a competent type-I IFN receptor (PyMT-Ifnar1−/− mice) revealed similar tumor growth kinetics for both genotypes (Fig. 1A and B), leading us to conclude that endogenous IFN signaling was not a key regulator of primary tumor initiation or growth of PyMT-driven tumors. Other groups focusing on tumor immunity have also observed a lack of PyMT primary tumor responsiveness to immunologic challenge. One such report has shown that although PyMT lung metastasis can be contained by an effective CD8+ T-cell response, T-cell immunity at the primary site was likely dysfunctional due to intense immune suppression (25). These data illustrate an obvious challenge in promoting primary tumor

Figure 1. Ifnar1 deficiency results in accelerated bone metastasis in the spontaneous MMTV-PyMT-driven model of spontaneous tumorigenesis. A, time to palpable tumor development. B, time until maximum ethical tumor volume (1,500 mm³) in MMTV-PyMT WT (n = 30) and MMTV-PyMT Ifnar1−/− (n = 28) mice were assessed. Subsequent metastatic burden in the (C) spine, (D) femurs, and (E) lung of MMTV-PyMT WT (n = 22) and MMTV-PyMT Ifnar1−/− (n = 20) was quantified using RT-qPCR and expressed as RTB. *, P < 0.05 using Mann–Whitney U tests. Error bars, SEM. F, representative H&E and pan-cytokeratin (CK) stained femur sections from MMTV-PyMT WT and Ifnar1−/− mice at ethical tumor endpoint. Scale bars, 50 μm.
Orthotopic mouse mammary tumor models show distinct patterns of progression in hosts lacking a functional type-I IFN system

To assess the impact of a defective type-I IFN host response on transplantable orthotopic tumors in immunocompetent mice, we chose to use the highly lung metastatic 4T1 and weakly metastatic 66cl4 isogenic cell lines from the Miller series (18). These cell lines form primary tumors and metastasize reliably and predictably in Balb/c mice, allowing for appropriate design of experiments that can dissect the role of type I IFN signaling on tumor growth, metastasis, and immune activation.

Our experiments revealed that both models had vastly different patterns of progression depending on the type-I IFN signaling status of the host, and was independent of Ifnar1 expression (Fig. 6A) or intrinsic IFN sensitivity of either cell line (Supplementary Fig. S4A and S4B). Indeed, both cell lines were able to secrete substantial levels of IFNα upon stimulation (Supplementary Fig. S4C) and had similar basal levels of the key IFN-driven transcription factor, Irf7 (Supplementary Fig. S4D), suggesting that differences in IFN sensitivity or secretion could not account for the differential behavior of these cell lines in vivo. The 4T1 model followed a similar mode of progression to PyMT-driven tumors, with primary tumor growth (Fig. 2A) and lung metastasis (Fig. 2B and C) not affected by the IFN signaling status of the host. In contrast, the 66cl4 model showed a dramatic increase in lung metastasis in Ifnar1–/– mice, with a significant decrease in primary tumor growth (Fig. 2A). This finding is further underscored by the fact that these experiments were terminated due to the size of multiple primary tumors, rather than signs of metastatic distress. This reduced the time allowed for metastatic progression and no doubt reduced the frequency of bone metastasis. For this reason, we decided to move to transplantable tumor models to further study the impact of a lack of host type-I IFN signaling on metastasis and breast tumor immunity.
contrast, 66cl4 tumors appeared to be highly sensitive to endogenous type-I IFN signaling, such that primary tumor growth and lung metastasis were significantly increased in Ifnar1−/− mice (Fig 2A and B). As this acceleration of lung metastasis was also observed when experiments were controlled for primary tumor size (Fig. 2C), it appeared that Ifnar1−/− mice had a true defect in the antimetastatic response. Importantly, we found that both the 4T1 and 66cl4 cells more readily metastasized to the spine and femurs in Ifnar1−/− mice (Fig. 3A–C), further strengthening our finding of the critical nature of type-I signaling in regulating bone metastasis.

In light of multiple reports showing tumor immunity in these models (25, 26), and the fact that tonic IFN signaling is required to maintain normal hematopoiesis (27), our data indicate a role for the immune system in regulating metastatic spread. In support of this, the loss of hematopoietic Ifnar1 [by using chimeras made from WT recipients and normal (WT) or IFN-insensitive (Ifnar1−/−) bone marrow] reduced metastasis-free survival in the 4T1.2 model—an aggressive variant of the 4T1 line—in the absence of an impact on orthotopic tumor growth (Supplementary Fig. S1A and S1B). However, the specific immunologic mechanisms responsible for this survival advantage remain unclear.

Type-I IFNs promote NK-cell–mediated elimination of breast tumor cells

Homeostasis of the NK-cell compartment appears to be particularly dependent upon endogenous type-I IFN signaling, as reports have shown that Ifnar1−/− mice possess a relatively immature pool of these cells (28). Similarly, the dysfunction of the NK-cell compartment in Ifnar1−/− null mice has been implicated in the establishment of some tumors (16), and more recent data have proven the importance of these cells in preventing the outgrowth of experimental melanoma metastases (29). We therefore hypothesized that the differences in metastasis between WT and Ifnar1−/− hosts could be due in part to the ability of NK cells to recognize and eliminate the 4T1 and 66cl4 breast tumors.

We therefore measured NK-cell number and activation during progression of the transplantable tumors and found an indication that NK cells were responding to the developing tumor. Analysis of circulating NKP46+ NK-cell proportions in 4T1 and 66cl4 tumor–bearing mice indicated an early expansion of this population (Fig. 4A and D), with an increase from 4% to 6% 5 to 7 days after tumor cell inoculation. In support of this NK-cell response, we also observed differences in NK-cell CD69 expression (an early activation marker) and IFNγ restimulation potential in tumor-bearing hosts. Importantly, we found that Ifnar1−/− hosts had...
significantly diminished levels of both of these markers in comparison with WT 4T1–bearing counterparts (Fig. 4B and C). A similar (although not significant) trend was also observed in the 66cl4 model (Fig. 4E), though NK cells from WT tumor–bearing mice were still able to produce more IFNγ than their naïve counterparts. Together, these data indicate that WT NK cells were responding to the developing cancer but that this response was impaired in Ifnar1−/− hosts.

In addition to this apparent heightened NK-cell activity in WT tumor–bearing mice, we found that they were also capable of killing mammary tumor lines in vitro. Cytotoxicity experiments revealed that in vitro–activated NK cells purified from WT but not Ifnar1−/− mice (Fig. 5A) or mice administered an Ifnar1–blocking antibody (Fig. 5C) could effectively eliminate 4T1 tumor cells, showing a clear dependency on type-I IFN signaling in arming the cytotoxic response against this line. In contrast, similar experiments with the 66cl4 line showed an apparent resistance to cytotoxicity, irrespective of whether the NK cells were derived from WT or Ifnar1−/− hosts (Fig. 5B). Although these data were somewhat at odds with the relative metastatic ability of each tumor model, these findings highlight the critical importance of using the cell type under investigation as the target in such cytotoxicity assays. Many other studies likely miss these nuances by correlating NK-cell cytotoxicity with highly susceptible target cells (such as the Yac-1 line we used as experimental controls in Fig. 5B). In addition, cytotoxicity assays reported in the literature commonly use high concentrations of cytokines, such as IL2, to expand and activate the population beyond what would be physiologically reasonable, masking the impact of IFN signaling and target-specific sensitivities to NK-cell attack. Indeed, under these hyperactivating conditions, we have previously observed no difference in cytotoxic ability between NK cells

Figure 4. Impaired functionality of Ifnar1−/− NK cells after orthotopic injection of 4T1 or 66cl4 cells. A–C, female age matched WT (n = 5) and Ifnar1−/− (n = 5) mice were injected with 1 × 10^6 4T1 or (D and E) 1 × 10^6 66cl4 cells into the 4th mammary gland. On indicated days after tumor cell inoculation, blood samples were taken and mononuclear cells were immunostained to reveal (A and D) the proportion of circulating (NKp46+ ) NK cells, (B) activated (NKp46+/CD69+) NK cells, and (C and E) NK-cell IFNγ production (NKp46+/IFNγ+cells) by flow cytometry. Naïve NK-cell proportions shown in A are also shared with D. Data are represented as the proportion of peripheral blood lymphocytes (PBL), and each experiment was repeated twice. *, P < 0.05; **, P < 0.005; ***, P < 0.0005 using Student t tests. Error bars, SEM.
derived from WT and Ifnar1−/− mice, and even lysis of the apparently resistant 66cl4 line (Supplementary Fig. S2). Therefore, our approach of dosing mice with poly(I:C) (a well-characterized IFN-agonist; ref. 30), allowing in vivo activation, and using NK cells immediately after isolation were ideal experimental conditions to examine type-I IFN-mediated cytotoxicity against our tumor lines.

Given the apparent susceptibility of the 4T1 cell line to NK-mediated cytotoxicity in vitro, we conducted NK-cell therapy experiments to understand whether adoptive transfer of this immune cell type alone could restrain metastatic spread. Ifnar1−/− hosts were chosen due to the enhanced rate of bone metastasis seen in these mice and to negate the contribution of endogenous IFN signaling to the immune system. 4T1 tumor–bearing hosts were then intravenously infused with NK cells purified from poly(I:C) stimulated WT or Ifnar1−/− donors at two time points prior to tumor resection and twice after surgery. In accordance with our other 4T1 tumor growth data (Fig. 2A), the infusion of NK cells from either donor genotype did not affect primary tumor growth (Fig. 5D). In order to provide a reference for data distribution, we overlaid metastasis data gathered from these NK-cell transfer experiments (Fig. 5E and F, black points) on our previous 4T1 metastasis data (from Figs. 2B and 3A, gray points). These comparisons clearly show that the transfer of WT or

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<th>Target cell lysis (%)</th>
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**Ifnar1−/− NK :**
- 4T1
- 66cl4

**WT NK :**
- 4T1
- 66cl4

**Target cell lysis (%)**
- 50
- 25
- 12.5
- 6.25
- 3.125
- 1.5625

**Effector : Target ratio**
- -10
- 0
- 10
- 20
- 30
- 40
- 50
- 60
- 70
- 80
- 90
- 100

**Primary tumor weight (g)**
- 0
- 0.1
- 0.2
- 0.3
- 0.4
- 0.5
- 0.6
- 0.7
- 0.8
- 0.9
- 1
- 1.2
- 1.4
- 1.6
- 1.8
- 2

**Lung metastasis**
- WT NK cell transfer
- Ifnar1−/− NK cell transfer
- Whole animal

**Spine metastasis**
- WT NK cell transfer
- Ifnar1−/− NK cell transfer
- Whole animal

**P = 0.08**
Figure 6. NK cells initiate 66cl4 tumor cell apoptosis through agonism of the death-receptor pathway. A, 66cl4 and 4T1 tumor cells were analyzed for the expression of Balb/c MHC class-I molecules (H2D(d) and H2K(d)) as well as ligands for activating NK-cell receptors (CD155, MULT1, Rae-1, and H60) and the type-I IFN receptor (Ifnar1) by flow cytometry. Histograms are representative of two independent experiments. B, in vivo-stimulated NK cells (250 μg i.p. poly(I:C), 48 hours prior to NK isolation) from WT and Ifnar1−/− mice were purified and cocultured with calcein-AM-labeled 66cl4, 4T1, or Yac-1 cells for 18 hours. Target cell lysis was quantified by detecting calcein release into the supernatant (pooled NK cells from 2 mice were used in experiments with 3 technical replicates, repeated twice). Error bars, SEM of all replicates. C, unlabeled 66cl4 cells were cocultured with in vivo-stimulated WT or Ifnar1−/− NK cells as described in B. After 18 hours, the remaining cells were stained with TO-PRO-3 and Annexin-V–FITC to reveal the apoptotic population (TO-PRO-3+/Annexin-V− cells) by flow cytometry. Data are representative of two independent experiments.

Ifnar1−/− donors did not affect metastatic burden in the lung (Fig. 5E); however, there was a clear reduction in spine metastasis observed in Ifnar1−/− mice infused with activated WT NK cells (Fig. 5F) that correlated with reduced spine metastasis in WT animals (Figs. 3A and Fig. 5F gray points). Although this impact on spine metastasis did not reach the traditional cutoffs of significance using standard statistical tests (perhaps due to the small experimental cohort), it does suggest a role for NK cells in conferring antimetastatic protection in the bone.

We next investigated the apparent paradox between NK susceptibility and metastatic potential. As NK-cell recognition is known to depend on the net signaling outcome from both activating and inhibitory receptors (31), we wondered whether the expression of their cognate ligands on the tumor cell surface would correlate with our cytotoxicity assays. We found that levels of MHC class-I molecules recognized by Ly49 family of inhibitory NK-cell receptors were expressed at higher levels on 66cl4 cells relative to 4T1 cells (Fig. 6A). Although the activation and release of cytotoxic granules from NK cells are known to be dependent upon multiple signaling inputs (32), it is plausible that this difference in MHC class-I contributed to the susceptibility of 4T1 but not 66cl4 cells to rapid cytotoxic attack. However, we wondered whether 66cl4 cells could eventually succumb to NK-cell–mediated death if the period of coculture was increased. We therefore performed overnight cytotoxicity experiments and found that 66cl4 cells could indeed be killed by WT but not Ifnar1−/− NK cells (Fig. 6B), and that the proportion of apoptotic 66cl4 cells was greater when cocultured with WT NK cells (Fig. 6C). Although further elucidation of this mechanism was beyond the scope of this study, it is well known that NK cells can engage distinct modes of cytotoxicity that have variable kinetics of inducing target cell death (33). Together, the data presented indicate that type-I IFN signaling can have a broad impact on NK-mediated immune surveillance mechanisms.

Finally, we cannot rule out a contribution of the adaptive immune system to this IFN-linked antimetastatic response. Type-I IFNs are well known to support the expansion (34) and maintenance of activated CD8+ T cells (35). This, along with our observation of elevated T-cell number in WT hosts bearing 66cl4 tumors (Supplementary Fig. S3), would suggest some involvement of the adaptive immune system. Interestingly, one very recent study has integrated our findings by reporting that NK cells may contribute to tumor immunity by promoting antigen cross-presentation (36). This suggests that some of the roles NK cells play during antitumor immunity may be independent of their cytotoxic function. Thus, in addition to closer examination of the adaptive immune response in these models, future studies will need to further investigate the relative contribution of NK cells to the global antimetastatic response—perhaps through the use of recently published models of innate immunodeficiency (29).

Concluding Remarks

Models of spontaneous breast cancer bone metastasis are rare. In this study, we have demonstrated that three non-bone metastatic breast tumor models can successfully colonize the bone in hosts with a defective type-I IFN pathway—underscoring the importance of IFN signaling during the antimetastatic response. An interesting area to investigate in the future will be how the bone-resident immunity (37) differs in relation to other metastatic sites, and why tumors that induce large numbers of circulating suppressive cells fail to metastasize to this organ without the loss of type-I IFN signaling. Indeed, myeloid-derived suppressor
cell (MDSC) accumulation has been implicated with "premetastatic niche" formation in the lungs, which may explain why lung metastasis was not consistently enhanced by a loss of IFN signaling. Also on the theme of site-specific immunity, it will be important to investigate whether differences in resident immune populations (such as the emerging picture of distinct NK-cell populations; refs. 39, 40) are responsible for the discrete patterns of metastasis of some cancers. Finally, our data show a potential role for NK cells to engage in the antimetastatic immune response, though it is unclear whether this population can gain sufficient stimulation during cancer progression to effectively eliminate disseminated cells. As some studies have reported a general decrease in NK functionality during breast tumor progression (41), IFN-based therapies aimed at reactivating these cells may prove beneficial as novel antimetastatic therapies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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