Disruption of IFN-I Signaling Promotes HER2/Neu Tumor Progression and Breast Cancer Stem Cells

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Abstract

Type I interferon (IFN-I) is a class of antiviral immunomodulatory cytokines involved in many stages of tumor initiation and progression. IFN-I acts directly on tumor cells to inhibit cell growth and indirectly by activating immune cells to mount antitumor responses. To understand the role of endogenous IFN-I in spontaneous, oncogene-driven carcinogenesis, we characterized tumors arising in HER2/neu transgenic (neuT) mice carrying a nonfunctional mutation in the IFN-I receptor (IFNAR1). Such mice are unresponsive to this family of cytokines. Compared with parental neu+/− mice (neuT mice), IFNAR1−/− neu+/− mice (IFNAR-neuT mice) showed earlier onset and increased tumor multiplicity with marked vascularization. IFNAR-neuT tumors exhibited deregulation of genes having adverse prognostic value in breast cancer patients, including the breast cancer stem cell (BCSC) marker aldehyde dehydrogenase-1A1 (ALDH1A1). An increased number of BCSCs were observed in IFNAR-neuT tumors, as assessed by ALDH1A1 enzymatic activity, clonogenic assay, and tumorigenic capacity. In vitro exposure of neuT mammospheres and cell lines to antibodies to IFN-I resulted in increased frequency of ALDH+ cells, suggesting that IFN-I controls stemness in tumor cells. Altogether, these results reveal a role of IFN-I in neuT-driven spontaneous carcinogenesis through intrinsic control of BCSCs.

Introduction

Type I interferon (IFN-I) are pleiotropic cytokines that exert multiple biological effects in viral infections and cancer (1, 2). Injection of IFN-I-blocking antibody resulted in enhanced transplanted tumor growth, suggesting that endogenous IFN-I controls tumor development and progression in immunocompetent mice (3). Endogenous IFN-I functions in cancer immune surveillance (4) and in the innate recognition of tumors by several immune cells (5).

Studies in mouse models show that IFN-I inhibits growth of transplanted tumors and exerts biological effects on immune cells, including NK cells, dendritic cells (DCs), T, and B cells (2). Thus IFN-I links innate immunity and development of the anti-tumor immune response (5).

HER2 is an oncogene overexpressed in 15% to 30% breast cancers (6). HER2+ breast cancers are characterized by a molecular signature distinguishing these cancers from other breast cancer types (7). HER2 overexpression is a negative prognostic factor, associated with poorly differentiated, high-grade tumors, shorter disease-free and overall survival (6). Mice engineered to express an activated rat neu gene (Erbb2, ortholog of HER2) mimic most of the features observed in human HER2+ breast cancers (8, 9). In fact, during the first postnatal weeks, foci of atypical hyperplasia can be observed in mammary glands of neu transgenic mice. Gradually, these foci coalesce to form large carcinomas in situ, which progress to invasive carcinomas (10).

Despite the extensive characterization of IFN signaling in tumor biology and studies on the mechanisms behind neu-driven tumorigenesis, little is known about endogenous IFN-I signaling in neu transformation and progression and in spontaneous carcinogenesis. Here, we studied neu-driven tumorigenesis in mice unresponsive to IFN-I by generating a mouse...
strain transgenic for HER2/neu and lacking the IFN-I receptor IFNAR1 (IFNAR-neuT mice).

Materials and Methods

Mice

NeuT and IFNAR-neuT transgenic mice were generated on 129Sv background and bred in the animal facility of the Department of Oncology and Molecular Medicine at the Istituto Superiore di Sanità (Rome, Italy). Pure neuT and IFNAR-neuT mice were obtained by at least 12 backcrosses of BALB-neuT transgenic males (kindly provided by Dr. Guido Forni, University of Torino, Torino, Italy) with C57Bl/6 IFNAR1 transgenic males (kindly provided by Dr. Guido Forni, University of Torino, Torino, Italy) and bred in the animal facility of the Department of Oncology and Molecular Medicine at the Istituto Superiore di Sanità (Rome, Italy). Pure neuT and IFNAR-neuT mice were maintained in accordance with the European Community.

Cell lines and mammosphere culture

One tumor cell line was isolated from a spontaneous mammary tumor developed in a neuT virgin female, stabilized in vitro and named 676-1-25 as previously described (11). N202.1A and N202.1E are cloned cell lines derived from an in vitro tumor cell line isolated from a spontaneous mammary tumor developed in a neuT virgin female, stabilized in vitro and named 676-1-25 as previously described (11). Both cell lines were kindly provided by Dr. Claudia Curcio ("Gabriele d’Annunzio University, Chieti, Italy) and tested by flow cytometric analysis for HER2 expression. All cells were cultured in RPMI 1640 supplemented with 10% FCS. Cells were authenticated by morphology and growth in vitro and in vivo and routinely tested for mycoplasma; cells were used for experiments within three passages after thawing master cell bank aliquots stored in liquid nitrogen.

Mammary gland tissues were collected at the designed time points for flow cytometric analysis, in vitro clonogenic assays or in vivo tumorigenic assay. Briefly, tumor samples were freed from hemorrhagic and necrotic parts, washed in phosphate-buffered saline (PBS), minced with scissors, and digested in serum-free medium supplemented with collagenase (Sigma Blend F, Sigma-Aldrich) 400 U/mL and DNAse type I 200 U/mL at 37 °C for 30 minutes. After incubation, 5 mL RPMI 10% FCS EDTA 2 mmol/L was added, and cells were then filtered in cell strainers and washed twice in PBS.

For mammosphere culture, cells recovered from transgenic mice (13) were cultured at clonal density in serum-free medium supplemented with 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (bFGF). Ultralow attachment cell culture flasks were used. The medium was replaced or supplemented with fresh growth factors once a week until cells started forming floating aggregates. Cultures were expanded by mechanical dissociation of spheres, followed by replating of both single cells and residual small aggregates in complete fresh medium.

Immunophenotypic analysis

Immunophenotypic analysis of cells isolated from spleens, lymph nodes, bone marrow, blood, and mammary carcinomas of mice was carried out as follows: cell suspensions were treated with lysis buffer (140 mmol/L NaCl, 17 mmol/L Tris HCl, pH 7.2) to eliminate red blood cells and then stained with the following fluorescently labeled mAbs: anti-CD45 (30-F11), anti-CD11c (N418), anti-CD3 (145-2C11), anti-CD8a (53-6.7), anti-CD25 (PC61.5), anti-CD357/GITR (DTA-1), anti-Foxp3 (FKH-16s) from eBioscience; anti-CD4 (GK1.5), anti-Ly6C (AL-21), anti-Ly6G (1A8) from BD Pharmingen; anti-CD11b (M1/70), anti-CD19 (6D5), anti-Gr-1 (RB6-8C5), and anti-mPDCA1 (IF05-1C24.1) from Miltenyi Biotec. For detection of HER2/Neu expression in tumors, cells isolated from mammary tumors were stained with PerCP labeled anti-CD45 coupled to anti-HER2/Neu (NEU 7.16.4, Calbiochem) followed by mouse Alexafluor488 and by propidium iodide (PI). The percentage of HER2/Neu expression was determined in the PI-CD45− cell population (bona fide tumor and stromal cells). For the analysis of putative CSC markers, tumor cells isolated from 24-week-old transgenic mice as described above were stained with the following fluorescently labeled mAbs: anti-CD4 (MEC.13.3), anti-CD140a (APAS) from Biolegend; anti-Ly6A/Sca1 PE (D7), anti-CD133 APC (13A4); anti-CD24 (M1/69) from eBioscience; anti-CD44 (IM7), anti-CD44f (GoH3), anti-CD61 (2C9.62) from BioLegend; anti-LGR4 (GPR49) from Abgent; and anti-EpCAM (G8.8) from Santa Cruz Biotechnology. For the immunophenotypic analysis of spleen, lymph node, bone marrow, blood, and mammary carcinomas, cell populations were defined as follows: CD4 T cells: CD45 CD3 CD19 CD4 CD8; CD8 T cells: CD45 CD3 CD19 CD4 CD8; B cells: CD45 CD3 CD19; Treg cells: CD45 CD3 CD4 CD8 CD25 Foxp3 GITR; MDSC: CD45 CD11b−Gr-1−CD124/IlaR−; Mo-MDSC: CD45 CD11b−Ly6C Ly6G−; Gr-MDSC: CD45 CD11b−Ly6C Ly6G+; pDC: CD45 mPDCA1 CD11b CD11c+; All samples were run on a Gallios flow cytometer and analyzed with Kaluza Analysis Software (Beckman Coulter).

In vitro clonogenic assays and tumorsphere formation assays

Soft agar colony-forming assays were carried out as previously described (13). For tumorsphere formation assay, cells isolated from tumors were plated in ultra-low attachment six-well plates (Corning) at a density of 10,000 cells/mL in serum-free medium supplemented with 20 ng/mL epidermal growth factor (EGF, Sigma-Aldrich), 10 ng/mL basic fibroblast growth factor (b-FGF,
Cells were cultured under 5% CO₂ at 37°C for 1 week.

**In vivo tumorigenesis assays**

Tumors were excised from 23-week-old neuT and IFNAR-neuT, dissociated and the cells were counted and resuspended in a mixture of PBS and Matrigel (1:1). A total of 1 x 10⁶ cells were injected subcutaneously into the right flank of each mouse or ortho/itopically injected into the right fat pad. At day 30, tumors were excised and prepared for immunohistochemistry analysis (see below).

**Morphological analysis of the mammary glands**

Groups of four mice were sacrificed at the indicated times, and mammary tissue was processed as previously described for histologic, immunohistochemical, and whole-mount analysis (14). For whole-mount preparation, the pelt of euthanized transgenic and control mice was fixed overnight in 10% neutral buffered formalin. The mammary fat pads were scored into quadrants and gently scraped from the pelt. The quarters were immersed in acetone overnight and then rehydrated and stained in ferric hematoxylin (Sigma-Aldrich), dehydrated in increasing concentrations of alcohol, cleared with histo-lemon, and stored in methyl salicylate (Sigma-Aldrich). Digital photos were acquired with a Nikon Coolpix 995 (Nital SpA) mounted on a stereoscopic microscope (MZ6; Leica Microsystems) with a 0.63 objective giving a total magnification of ×6.3. The resolution was 1,600 x 1,200 pixels. Images were acquired with an Adobe Photoshop v. 6.0 graphic software (Adobe Systems). Mammary glands that exceeded the size of a single-imaging area were captured by photographing contiguous fields in a raster pattern. Each captured image was merged using the layer technique in Adobe Photoshop to form a single-composite picture for analysis. Spatial calibration was determined by photographing a 1-mm stage using the same parameters as those for image capturing of whole-mount preparations. The distance drawn on the 1-mm calibration image was divided by 1,000 to acquire a 1-mm stage. Digital images of 2 tumors per group (6 x 400 microscopic fields per tumor) in a double-blind fashion, and digital images of representative areas were taken. Vessels area (in pixels) was evaluated with Adobe Photoshop by selecting vessels with the lasso tool and reporting the number of pixels indicated in the histogram window.

**RNA isolation, labeling, and hybridization**

Inguinal mammary glands were isolated from 19-week-old mice. Tissues were submerged with RNA later (Qiagen), and stored at −20°C. For RNA extraction, the mammary glands were washed twice in PBS, immersed in TRIzol (QIAzol Lysis reagent). Tissues from individual mice were disrupted by 30° homogenization (Ika Homogenizer system T10), the resulting homogenate underwent high-speed centrifugation to remove particulate and total RNAs were eventually extracted by chloroform followed by isopropyl alcohol precipitation. Total RNAs were then spectrophotometrically quantified and quality was assessed by using the Agilent Model 2100 Bioanalyzer and the EukaryoteTotal RNA Pico Kit (Agilent Technologies). Low-quality RNAs were discarded. Forty nanograms of sample RNA underwent cRNA preparation and cyanin (Cy5) labeling by Low Input Quick Amp Labeling Kit Standard Dual Color (Agilent Technologies), according to the manufacturer's instruction. The same amount of universal reference mouse RNA (Stratagene) was processed and Cy3-labeled as reference control. Labeled amplified RNAs were purified with Qiagen's RNeasy mini spin columns and cRNA yields and specific activities were checked before hybridization. A total of 825 ng of Cy3-labeled, linearly amplified reference cRNA and 825 ng of Cy5-labeled, linearly amplified cRNA were fragmented, mixed, and cohybridized to mouse expression 4 x 44K v2 microarray slides (G4464A), in a final volume of 55 μL. Microarray slides were assembled into the appropriate chambers and placed in rotisserie in a hybridization oven set to 65°C for 17 hours. Slides were then disassembled and washed according to the manufacturer's instructions.

**Microarray data analysis**

Scanning and image file processing were performed with GenPix 4200A instrument (Axon Instruments). Images were analyzed using ScanArray Express software where spots with bad quality were omitted allowing the rejection of spurious signals due to artifacts. Slide quality assessment was performed according to the signal intensity obtained in the presence of Spike In samples. Data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE110350 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110350).

The resulting data files were analyzed via BRB ArrayTool (http://linus.nci.nih.gov/BRB-ArrayTools.html) developed at the National Cancer Institute, Biometric Research Branch, Division of Cancer Treatment and Diagnosis. The raw data set was filtered according to standard procedure to exclude spots with minimum intensity, arbitrarily set to <20 in both 20 in both monoclonal anti-CD105 (BD Pharmingen), followed by secondary antibody conjugated with Alexa 546 (Invitrogen, Life Technologies). Nuclei were stained with DRAQ5 (Alexis, Life Technologies). Image acquisition and analysis were performed using Zeiss LSM 510 META confocal microscope. The number and area of CD31+CD105+ vessels were evaluated on the digital images of 2 tumors per group (6 x 400 microscopic fields per tumor) in a double-blind fashion, and digital images of representative areas were taken. Vessels area (in pixels) was evaluated with Adobe Photoshop by selecting vessels with the lasso tool and reporting the number of pixels indicated in the histogram window.
fluorescence channels. If the fluorescence intensity of one channel was >20 and that of the other below <20, the fluorescence of the low-intensity channel was arbitrarily set to 20. Spots with diameters <10 μm and flagged spots were also excluded from the analysis. The resulting database, consisting of 18,361 transcripts, was used for further statistical analysis. Filtered data were then normalized in log space using locally weighted linear regression (Lowess). All statistical analyses were done using the log_2-based ratios. Supervised class comparison between sample classes was performed with the BRB ArrayTool using the random-variance model with parametric P value threshold set to 0.005. Significantly differentially expressed genes resulting from the analysis were further analyzed using cluster and TreeView software. Gene ratios were average corrected across experimental samples and displayed according to the central method for display using a normalization factor as recommended by Ross (15). Gene function and classification was assigned by means of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/). Overlaps with published gene signatures were assessed using the Molecular Signature Database (MSigDB; http://software.broadinstitute.org). To evaluate the prognosis of gene signatures in breast cancer patients, the dataset publicly available on the KM plotter website was used (www.kmplot.com; 17).

Aldehyde assay by FACS

The ALDEFLUOR kit (StemCell Technologies) was used to analyze the population with a high ALDH enzymatic activity. Cells obtained from freshly dissociated breast tissue or mammosphere cultures were first surface-stained with antibodies directed against lineage markers (Ter119, CD31, CD140a, and CD45), then resuspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 μmol/L per 1 × 10^6 cells) and incubated for 40 minutes at 37°C. As a negative control for each sample of cells, an aliquot was treated with 50 mmol/L diethylaminobenzaldehyde (DEAB), an ALDH inhibitor. Cells were then labeled with propidium iodide (PI) and analyzed by flow cytometry. Positivity for ALDH was assessed in cells negative for PI and lineage markers expression. DEAB-treated samples served as controls for background fluorescence.

Statistics and reproducibility

Results are represented as means ± SEM. For group comparisons, we used nonparametric two-tailed Mann–Whitney–Wilcoxon U test. For free-survival analysis, Kaplan–Meier plots and significance with P log-rank test were used. All statistics were calculated with Openstat and Microsoft Excel software. The experiments based on images (Whole-Mount analysis, FACS, IF, H&E staining) were repeated at least 3 times and representative images are shown. Microarray data statistical analysis, as detailed in the microarray section, was performed using BRB ArrayTool (http://linux.nci.nih.gov/BRB-ArrayTools.html). For supervised analysis, the random variance model was used, and the parametric P value threshold was set to 0.005. For gene ontology analysis, biological process classes were ranked according to the −log(P) of the modified Fisher test. The overlaps with gene signatures present in the MSigDB were calculated by the false discovery rate of q values and by the ratio between the number of genes of the two sets.

Results

IFN-I signaling disruption accelerates neuT-driven tumorigenesis

To examine the role of endogenous IFN-I signaling on mammary tumorigenesis, we generated a mouse strain carrying the HER2/neu oncogene and lacking a functional IFNAR1. 129Sv IFNAR1^+/− mice (18) were backcrossed with Balb/C HER2/neu transgenic mice for more than 12 generations, until a colony of neu^+/− IFNAR1^+/− mice on a 129Sv background was obtained (IFNAR-neuT mice). Control 129Sv neu^+/− mice expressing functional IFN-I receptor gene (IFNAR1^+/+) were also generated for comparison (negT mice). The results of tumor growth monitoring in both strains revealed that, despite nonstatistically significant earlier onset of first palpable lesion (Fig. 1A), IFNAR-neuT mice showed significantly higher mean tumor multiplicity (P < 0.01; Fig. 1B), achieving tumor formation in all 10 glands approximately 4 weeks earlier as compared with control neuT mice. A comparative whole-mount microscopy analysis of the mammary glands showed similar progression from hyperplastic lesions to large microscopic lesions (equivalent to in situ carcinomas) in neuT and IFNAR-neuT mice until the 15th week of age (Fig. 1C). In contrast, the progression to invasive solid carcinomas was accelerated in IFNAR-neuT mice with respect to controls. In fact, by week 19, IFNAR1^+/−, but not parental NeuT mice, displayed enlarged and partially clumped lesions, and, by week 26, large invasive carcinomas occupied the whole mammary gland, whereas control mice showed fewer invasive carcinomas (Fig. 1C). Hematoxylin–eosin staining of tumor sections confirmed larger lesions in IFNAR-neuT than in neuT mice from week 15 to 26, although we observed no differences in tumor differentiation grade at these stages (Fig. 1D). Altogether, these results indicate that endogenous IFN-I signaling disruption does not influence the early stages of tumor transformation but accelerates the growth of large invasive carcinomas without affecting differentiation grade. No significant increase in lung metastatic burden was observed in IFNAR-NeuT versus NeuT mice (80% vs. 69% of lungs with metastasis, respectively). Indeed, dissemination of cancer cells in HER2 transgenic mice has been reported to occur mostly from early lesions (7–9 weeks), before IFNAR-neuT tumor progression accelerates (19).

IFNAR-neuT tumors show increased angiogenesis but no difference in the immune infiltrate

Given that tumor vasculature sustains tumor growth, invasion, and metastasis, and that IFN-I has antiangiogenic properties (20), we analyzed tumor microvascular density by endothelial cell staining in neuT and IFNAR-neuT tumors of equivalent size (Fig. 2A). Although similar numbers of intratumoral vessels were found in both groups of carcinomas (Fig. 2B), tumor-associated vessels appeared to be 25% larger in IFNAR-neuT than in neuT lesions (P ≤ 0.05; Fig. 2C).

Disruption of IFN-I signaling did not alter the percentage and composition of tumor-infiltrating leukocytes in neuT lesions. No significant differences in the frequency of total infiltrating immune cells, tumor-infiltrating T lymphocytes, B cells, and both granulocytic and monocytic myeloid-derived suppressor cells (MDSC) were found in tumors of similar sizes arising in IFNAR-neuT and neuT mice (Fig. 2D and E). The two strains also shared a similar percentage of cells expressing the HER2/neu antigen (Fig. 2D). Similarly, immune cell phenotype was...
not altered in the blood, spleen, bone marrow, and lymph nodes of IFNAR1−/− mice, whether or not they were transgenic for HER2/neu (Supplementary Fig. S1). These findings suggest that the increased tumor growth observed in IFNAR-neuT mice may be related not to immunosurveillance but rather to the increased vessel size that resulted from absence of inhibition by endogenous IFN-I.

Mammary gland transcriptional program is affected by neuT carcinogenesis

We analyzed gene expression profiles of 19th week IFNAR-neuT and neuT inguinal mammary gland tumors. Although not all transgenic mice showed palpable tumors at 19 weeks (Fig. 1C), tumor masses were visible within those inguinal mammary glands chosen for the analysis at sacrifice. Mammary glands of 129svWT and 129sv IFNAR1−/− mice were also examined to better assess tumor-specific molecular changes.

Class comparison analysis among all groups (P ≤ 0.005) revealed 642 differentially expressed genes. A large set of genes was similarly expressed in neuT and IFNAR-neuT tumors (blue boxes in Fig. 3A), suggesting that some of the neuT-driven transcriptional changes are not affected by IFNAR status. We initially focused on the modulations shared by IFNAR-neuT and neuT tumors. To strengthen the statistical power of our analysis, we compared neuT tumors with nontransgenic samples (neuT and IFNAR-neuT vs. 129svWT and 129sv IFNAR1−/−; P ≤ 0.005).

Of the 742 differentially expressed genes, 513 were upregulated and 229 genes were downregulated in neuT tumor. Gene ontology analysis (Fig. 3B) revealed an overall overexpression, in neuT tumors, of genes related with response to stress (such as ATF3, ATF4, EGLN3, and PLSCR1), cell cycle (such as CDC25A, CDC25C, CDC8, and CDK1), immune genes (NMI, STAT2 and STAT5A), and epithelial cell proliferation (such as ITGB3, TGFβ2, and THBS1). Genes downregulated in tumor tissue mainly belonged to metabolic pathways such as those that drive the metabolic changes occurring upon transformation (Fig. 3B; ref. 21).

The genes upregulated by neuT tumorigenesis in our mouse model overlapped with published signatures of human breast
tumors (such as SOTIRIOU_BREAST_CANCER_GRADE_1_VS_3_UP) and with neut-specific signatures in mice (LANDIS_ERBB2_BREAST_TUMORS; MSigDB; http://www.broadinstitute.org/gsea/msigdb; Fig. 3C). Overall, gene expression profiling data of the spontaneous carcinogenesis in HER2/neu transgenic mammary glands suggested that shared molecular pathways could be responsible for the similarities observed in IFNAR-neuT and neuT tumors.

IFNAR-neuT tumors share gene expression profile with aggressive human breast cancers

To better characterize the effect of IFN-I disruption on 19-week-old HER2/neu spontaneous tumors, we compared expression profiles of neuT and IFNAR-neuT samples. We found 118 differentially expressed genes ($P \leq 0.005$; Fig. 4A), of which 60 were more expressed and 58 were less expressed in IFNAR-neuT versus neuT neoplasms. IFNAR-neuT tumors overexpressed transcripts encoding proteins involved in tumor invasiveness and epithelial-to-mesenchymal transition, such as Laminin C and fibronectin (22, 23), as well as ALDH1A1, a marker of cancer stem cells (CSC; ref. 24) and a negative prognostic factor for breast cancer patients (25). We, therefore, tested the prognostic value of the IFNAR-neuT overexpressed genes in breast cancer patients from the KM plotter data set (17). Although the gene signature has no prognostic value on relapse-free survival in the whole set of breast cancer patients, we observed adverse prognostic value in HER2$^+$ breast cancer patients on relapse-free survival and distant metastases-free survival (Fig. 4B–E). No prognostic value of the signature was observed by restricting the analysis according to other breast cancer subtypes except for progesterone-receptor negative and triple-negative breast cancers (Supplementary Fig. S2). Altogether, these results indicate that the IFNAR-neuT cancers are characterized by an expression profile that is observed in breast cancer patients with a worse prognosis, thus suggesting that the lack of IFN-I signaling increases tumor aggressiveness through pathways shared with certain subtypes of breast cancer.

Figure 2.

IFNAR-neuT mammary carcinomas showed larger vessels than neuT lesions but minimal changes of immune infiltrate. A, Confocal microscopy analysis of carcinoma samples stained with anti-CD31 and anti-CD105 (red) in neuT and IFNAR-neuT carcinomas. Nuclei are stained with DAPI. Single colors and merged images are shown. Quantification of the number of vessel (B) and vessel area (C) in neuT (blue bars) and IFNAR-neuT (red bars) carcinomas. Data are representative of three independent experiments. Results are represented as means ± SEM from 7 $\times$ 100 microscopic fields of 10 tumors. *, $P \leq 0.05$. D, Expression of HER2/neu and total infiltrating leukocytes (CD45$^+$) on spontaneous neuT (blue bars) and IFNAR-neuT (red bars) digested tumors, isolated from 26-week-old transgenic mice. E, Flow cytometry analysis of tumor-infiltrating cells in NeuT and IFNAR-NeuT carcinomas. Data are representative of three independent experiments and are expressed as mean percentages of the indicated immune cell subsets among gated CD45$^+$ total leukocytes in individual mice ± SEM. *, $P \leq 0.05$ ($n = 10$, Mann–Whitney U test).
IFNAR-neuT tumors show increased frequencies of breast CSC

The observed modulation of genes with an adverse prognostic value and involved in CSC, together with the reported correlation between HER2 overexpression and tumor-initiation capacity in vitro and in vivo (26, 27), prompted us to further investigate the role of ALDH1A1 in IFNAR-neuT mammary lesions.

Therefore, we analyzed the enzymatic activity of ALDH in lesions freshly isolated from IFNAR-neuT tumors as compared with the neuT counterparts. IFNAR-neuT lesions showed a higher percentage of ALDH⁺ cells as compared with neuT tumors (Fig. 5A and B). We did not observe altered expression of molecules previously reported to be CSC markers in HER2/neu transgenic mice (CD61, CD49f, CD24, Sca1, and CD44; refs. 28–30) or in other tumors (CD133, EPCAM, and LGR5; Supplementary Table S1; ref. 31).

To assess whether the increased presence of ALDH⁺ cells was paralleled by higher number of BCSCs in IFNAR-neuT tumors, we evaluated tumor cell self-renewal and tumorigenic capacity in vitro and in vivo. A soft agar clonogenic assay revealed that more colonies could be generated from IFNAR-neuT than from neuT tumor cells (Fig. 5C). The increased numbers of BCSCs were also tested by tumorigenic ability potential in vivo. Tumor cells isolated from IFNAR-neuT mice and orthotopically transplanted in the mammary gland of NOD/SCID/Gamma (NSG) mice led to tumor lesions that were palpable 7 days earlier than tumors derived from neuT mice (Fig. 5D). Moreover, IFNAR-neuT tumors grew faster, resulting in tumor nodules 6-fold larger than the control counterpart 30 days after transplant (Fig. 5D). Similar results were observed when freshly isolated tumor cells were transplanted subcutaneously in NSG mice (Supplementary Fig. S3), where IFNAR-neuT tumors became palpable sooner than neuT tumors and continued to grow faster. Histological analysis showed a similar morphology of neuT and IFNAR-neuT tumors in NSG xenografts (Fig. 5E), retaining the histological features of primary cognate tumors (Fig. 1D).

Altogether, these results indicate that, in the absence of functional IFN-I signaling, spontaneously arising neuT tumors are characterized by increased frequencies of BCSCs, endowed with increased self-renewal and tumorigenic capacity in vitro and in vivo.
In vitro exposure to anti–IFN-I increased numbers of ALDH⁺ cells in mammospheres

To analyze in vitro the effect of the lack of endogenous IFN-I signaling on neuT tumors, we cultured neuT tumor cells in nonadherent nondifferentiating conditions (mammospheres) and measured variations in the percentage of ALDH⁺ cells in the presence of a neutralizing anti–IFNα/β sheep immunoglobulin (anti-IFN; Fig. 6A and B; ref. 32). Mammospheres derived from neuT tumors showed more ALDH⁺ cells 24 hours after anti-IFN treatment (Fig. 6A and B). No modulation was observed after IFN-I neutralization in mammospheres derived from IFNAR-neuT tumors, which already showed more ALDH⁺ cells than their neuT counterparts (Fig. 6A and B). Anti-IFN treatment induced formation of bigger mammospheres after culturing in soft agar from neuT-derived tumor cells than from IFNAR-neuT-derived tumor cells, which are unable to respond to IFN-I (Fig. 6C). A similar increase in the number of cells expressing the CSC marker ALDH was observed in other neuT-adherent tumor cell lines when treated with anti-IFN (Fig. 6A and B). In particular, IFN-I neutralization elicited an increase in the frequencies of ALDH⁺ cells in the neuT-expressing cell lines 676 and N202.1A, isolated from tumors spontaneously developed in 129Sv and FVB transgenic mice, respectively (Fig. 6A and B). By contrast, no effect could be observed in N202.1E, the FVB-derived HER2/neu⁻ counterpart (Fig. 6A and B). These data suggest an involvement of IFN-I signaling in regulating the number of ALDH⁺ cells in neuT-expressing tumors in a manner dependent on the neuT oncogene.

Discussion

Here, we examined how the impairment of IFN-I signaling due to absence of a functional IFN-I receptor affects neuT tumorigenesis and its progression. Our results show enhanced tumor growth and altered tumor vasculature in IFNAR-neuT mice, but no differences in immune infiltrate composition. IFN-I signaling impairment affected the presence of BCSCs...
within tumor and altered expression of the CSC marker ALDH, thus highlighting a role for IFN-I in BCSC biology.

The results shown in the present paper imply that, in line with what observed in other in vivo mouse models by our group and others (33–36), endogenous IFN-I is constitutively expressed in neuT mice at low levels. Although below the limits of detection in our assay, endogenous IFN-I is in fact responsible for the effects on spontaneous tumors observed in the absence of a functional IFN receptor in vivo and in the presence of anti–IFN-I in vitro.

Given the pleiotropic activities of IFN-I, a variety of mechanisms, including the lack of antiproliferative effects on tumor cells and an indirect impact on tumor angiogenesis (37), may account for the observed acceleration of IFNAR-neuT versus neuT tumors. Rather than an increase in the number of vessels, we observed an enlargement of vessel area in IFNAR-neuT mice, which may improve permeability and diffusion rates, and thus improve oxygen and nutrient supply to the tumor.

IFN-I signaling disruption impairs immunosurveillance therefore boosting tumor growth (38). Some studies have shown that implantable and induced tumors grow faster in IFNAR1−/− mice due to the reduced ability of the immune system of these mice to develop an antitumor immune response (4). Our results did not reveal any change in immune cell composition between neuT and IFNAR-neuT mice at the tumor site and in the main lymphoid organs, thus suggesting that immunosurveillance mechanisms play a limited role in our setting. In transgene-driven tumorigenesis, tolerance mechanisms usually surge because dominant clones are selectively deleted in neuT transgenic mice (39). Therefore, under normal conditions, spontaneous HER2 tumors show limited immune cell infiltration (8), which can be potentiated by immunotherapies (11). In the absence of a spontaneous antitumor immune response (i.e., in neuT-tolerized mice), immunosurveillance dysfunctions are rarely observed. Therefore, we conclude that, in this setting, IFN-I–dependent immune impairment has a reduced role in tumorigenesis.

Enhanced expression of IFN-γ and other inflammatory mediators has been reported in IFNAR1−/− mice with respect to control animals (40), suggesting that redundant biological responses and immune mechanisms might compensate for lack of endogenous IFN-I responses in our neuT model.
Figure 6.
IFN-I neutralization in vitro increases the number of ALDH\(^+\) cells in mammospheres derived from neuT tumors and other HER2\(^+\) tumor cell lines. **A**, FACS profiles showing the effect of anti-IFN-I (50 U, IFN-neutralizing units of antibody/mL) 24 hours treatment on the percentage of ALDH\(^+\) cells in (i) mammosphere derived from neuT tumor; (ii) mammosphere derived from IFNAR-neuT tumor; (iii) neuT conventional tumor cells line isolated from neuT tumor; (iv) neu\(^-\)/+/C\(0\)/C\(0\)/C\(0\) tumor cells line isolated from HER2/neu FVB tumor-bearing mouse, and (v) neu\(^-\)/+/
/C\(0\)/C\(0\)/C\(0\) tumor cells line isolated from HER2/neu FVB tumor-bearing mouse. Representative plots for Aldefluor control (+DEAB, a specific ALDH inhibitor, left) and test (right) samples are shown for each condition. **B**, Mean percentage of ALDH\(^+\) cells in control (white bar) and test (gray bar) samples for anti-IFN-I-treated samples versus untreated (NT) or mock-treated controls. Data are representative of at least 3 independent experiments, all performed with four replicates per group. Error bars represent standard errors and statistical differences are indicated \(^*\), \(P \leq 0.01\) (\(n = 4\), Mann–Whitney U test). **C**, Effect of anti-IFN-I (50 U/mL) for 24 hours treatment on the morphology of neuT and IFNAR-neuT mammospheres, as shown by phase-contrast microscopy. Data representative of three different experiments. Magnification, 20x.
Downstream mechanisms thought to be controlled by HER2 during neoplastic transformation (41) include cell-cycle deregulation, loss of cell polarity and cell adhesion, induction of invasive phenotype, and cellular metabolism. These processes are surely affected by HER2/neu (41). Our analysis of the gene expression data showed induction of transcripts involved in regulation of cell cycle, apoptosis, and metabolic pathways compared with nontransformed mammary gland. The observed molecular changes are representative of oncogenic signatures reported in other in vitro and in vivo breast cancer settings. Most of the HER2-transformation-induced changes were shared between neuT and IFNAR-neuT tumors, thus corroborating the impact of carcinogenesis on mammary gland transcriptional program. Nonetheless, IFNAR-neuT tumors showed upregulation of a limited set of transcripts including, among the others, genes involved in tumor aggressiveness and epithelial-to-mesenchymal transition, such as laminin C and fibronectin, as well as ALDH1A1. This latter enzyme is a marker of stemness in several solid tumors including breast cancer (42) and is associated with higher invasiveness and poor prognosis (43). An increased expression of ALDH1A1 in normal and malignant breast carcinoma cell lines has been reported in response to HER2 overexpression, thus implying that HER2 carcinogenesis and tumor invasion involve the modulation of the stem cell compartment, and that the expression of ALDH1A1 can play a role in this process (26). The signature of overexpressed transcripts in IFNAR-neuT lesions showed an adverse prognostic value in breast cancer patients, suggesting that the downstream pathways leading to increased aggressiveness observed in IFNAR-neuT tumors are shared with human breast cancers.

Many cytokines are known to regulate BCSC homeostasis (44). Exogenous IFNα exerts antiproliferative and proapoptotic effects on CSCs when administered per se or in combination with epigenetic drugs (45). The CSC-inducing activity of CD95 is mediated by STAT1 and involves IFN-Ι pathway (46), and BCSC biology depends on the mir-199-LCOR-IFN-Ι axis (35). In fact, BCSCs through the overexpression of mir-199 and the consequent downregulation of LCOR, escape IFN-Ι signaling, thus leading to a more aggressive tumor phenotype. Our results imply that basal concentrations of endogenous IFN-Ι act as negative regulators of BCSC homeostasis in neuT mice. IFNAR-neuT tumors showed more BCSCs as assessed not only by ALDH1A1 cells expression and enzymatic activity, but also by clonogenic assays and in vivo tumorigenicity after orthotopic transplant in NGS mice. The possibility that BCSC homeostasis is affected by IFN-Ι signaling rather than being mediated by immune-related effects is supported by the finding that in vitro exposure to IFN-Ι–blocking antibody increased ALDH expression in neuT-expressing BCSC and oncogene-positive tumor cell lines. This effect was visible on IFN-Ι-competent cells and absent in their knockout counterparts. No alteration of the ALDH+ compartment was induced by IFN-Ι-neutralization on breast tumor cells negative for HER2/neu, thus suggesting that the oncogene can be involved in IFN-mediated regulation of ALDH expression. CSCs represent key players in malignancy and are responsible for reduced success of cancer therapies (47–49). Increased expression of the detoxifying enzyme ALDH1A1 may provide CSCs a means to resist chemotherapy, particularly cyclophosphamide (50). We show here that the disruption of IFN-Ι-signaling affects ALDH expression in tumor cells and BCSCs, indicating a role of endogenous IFN-Ι in the restriction of BCSCs development and phenotype. Other than just highlighting a role of IFN-Ι-signaling in BCSC biology, these findings suggest involvement of endogenous IFN-Ι in breast as well as other cancers. The clinical efficacy of anti-HER2-specific immunotherapy combined with trastuzumab is dependent on the IFN-Ι pathway without requiring lymphocyte-mediated cytotoxicity (51). It would be of interest to evaluate whether the IFN-Ι contribution to trastuzumab efficacy also involves BCSC biology or chemo-sensitivity, perhaps through modulations of cancer cell ALDH expression in patients. Activation of IFN-induced genes in residual tumor cells is an early marker of chemotherapy responsiveness in HER2+ breast cancers (52). Thus, our results provide a rationale for using these cytokines in HER2+ breast cancer patients, perhaps in combination with other treatments to lower the BCSC fraction.

Our results may also suggest mechanisms by which IFN can control tumor development and progression in other human malignancies. As an example, in chronic myeloid leukemia (CML), IFNα therapy is receiving renewed interest due to its long-term efficacy, suggesting that these cytokines can act not only as immunomodulators, but also can restrain CML progenitors responsible for tumor relapse (53). Even in this setting, IFNα may regulate ALDH expression and thus might restrict CSC population.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


