**Poor Response to Neoadjuvant Chemotherapy Correlates with Mast Cell Infiltration in Inflammatory Breast Cancer**

Sangeetha M. Reddy1,2, Alexandre Reuben3,4, Souptik Barua5,6, Hong Jiang3, Shaojun Zhang7, Linghua Wang7, Vancheswaran Gopalakrishnan5, Courtney W. Hudgens8, Michael T. Tetzlaff8,9, James M. Reuben10,11, Takahiro Tsujikawa12,13, Lisa M. Coussens12, Khalida Wani8, Yan He8, Lily Villareal11, Anita Wood11, Arvind Rao6,14, Wendy A. Woodward11,15, Naoto T. Ueno1,11, Savitri Krishnamurthy11,16, Jennifer A. Wargo3,7, and Elizabeth A. Mittendorf17,18,19

**Abstract**

Our understanding is limited concerning the tumor immune microenvironment of inflammatory breast cancer (IBC), an aggressive form of primary cancer with low rates of pathologic complete response to current neoadjuvant chemotherapy (NAC) regimens. We retrospectively identified pre-treatment (N = 86) and matched posttreatment tissue (N = 27) from patients with stage III or de novo stage IV IBC who received NAC followed by a mastectomy. Immune profiling was performed including quantification of lymphoid and myeloid infiltrates by IHC and T-cell repertoire analysis. Thirty-four of 86 cases in this cohort (39.5%) achieved a pathologic complete response. Characterization of the tumor microenvironment revealed that having a lower pretreatment mast cell density was significantly associated with achieving a pathologic complete response to NAC (P = 0.004), with responders also having more stromal tumor-infiltrating lymphocytes (P = 0.035), CD8+T cells (P = 0.047), and CD20+ B cells (P = 0.054). Spatial analysis showed close proximity of mast cells to CD8+ T cells, CD163+ monocytes/macrophages, and tumor cells when pathologic complete response was not achieved. PD-L1 positivity on tumor cells was found in fewer than 2% of cases and on immune cells in 27% of cases, but with no correlation to response. Our results highlight the strong association of mast cell infiltration with poor response to NAC, suggesting a mechanism of treatment resistance and a potential therapeutic target in IBC. Proximity of mast cells to immune and tumor cells may suggest immunosuppressive or tumor-promoting interactions of these mast cells.

**Introduction**

Inflammatory breast cancer (IBC) is an aggressive form of the disease characterized by dermal lymphatic blockage by the tumor mass or tumor emboli, leading to the clinical appearance of inflammation on initial presentation (1). Locally advanced IBC is routinely treated with neoadjuvant chemotherapy (NAC) followed by surgery and radiotherapy; however, these patients experience low pathologic complete response (pCR) rates and Women’s Hospital, Boston, Massachusetts. 18Breast Oncology Program, Dana-Farber/Brighton and Women’s Cancer Center, Boston, Massachusetts.

**Note:** Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

N.T. Ueno, S. Krishnamurthy, J.A. Wargo, and E.A. Mittendorf contributed equally to this article.

**Corresponding Authors:** Elizabeth A. Mittendorf, Brigham and Women’s Hospital, Breast Oncology Program, Dana-Farber/Brighton and Women’s Cancer Center, Yawkey Center, Suite 1220, 450 Brookline Drive, Boston, MA 02115. Phone: 617-582-9980; E-mail: emittendorf@bwh.harvard.edu; Jennifer A. Wargo, Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 1484, Houston, TX 77030. Phone: 713-745-1553; E-mail: jwargo@mdanderson.org; Savitri Krishnamurthy, Department of Pathology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 0053, Houston, TX 77030. Phone: 713-794-5625; E-mail: skrishna@mdanderson.org; and Naoto Ueno, Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 1354, Houston, TX 77030. Phone: 713-792-8754; E-mail: nueno@mdanderson.org

doi: 10.1158/2326-6066.CIR-18-0619
©2019 American Association for Cancer Research.

www.aacrjournals.org
 (~15%) (2) and poor survival outcomes compared with stage matched non-IBC cases (3, 4). De novo stage IV patients may also receive NAC followed by aggressive local therapy for palliative purposes given the significant breast discomfort associated with IBC. Investigations are ongoing into therapeutic benefit of this strategy that emphasizes local disease control (5, 6). Unfortunately, targeted genomic profiling has provided limited insight into what distinguishes IBC from non-IBC, and molecularly targeted trials have shown limited success.

Immunotherapeutic agents such as monoclonal antibodies targeting immune checkpoints have revolutionized treatment of many types of solid tumors; however, they have shown modest efficacy thus far in breast cancer (7). There remains significant interest in evaluating the role of immune-checkpoint blockade in breast cancer, including IBC. Available data have shown that IBC is associated with upregulated inflammatory pathways (e.g., NF-κB, JAK/STAT, IL6, COX-2, ref. 8) and that defects in antitumor immunity may contribute to the tumor-igenesis and evolution of IBC (9, 10). In contrast, cytotoxic T-cell responses and associated exhaustion markers such as PD-L1 have been correlated with favorable outcomes in IBC (11, 12). Unfortunately, the majority of published data are based on gene-expression profiling. Therefore, there is a critical need for comprehensive morphologic characterization of immunologic aspects of the IBC tumor microenvironment (TME) and the relation to clinical outcomes.

To address this gap in understanding, this study was undertaken to morphologically characterize immunologic aspects of the IBC TME in a prospectively collected cohort of treatment-naïve locally advanced and de novo stage IV IBC patients, and to correlate these features with pathologic response to NAC. In addition to confirming biomarkers of response previously reported in non-IBC patients (tumor-infiltrating lymphocytes, CD8+ T cells), we identified mast cells, a critical regulator of several inflammatory conditions, as strongly associated with poor treatment outcomes. Mast cells represent a potential therapeutic target against which pharmacologic agents already exist, making translation readily accessible for future clinical studies. In addition, these findings provide additional evidence for the "inflammatory" underpinnings of IBC and its poor prognosis.

Materials and Methods

Study oversight

This study was approved by the MD Anderson Cancer Center Institutional Review Board. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki, and the protocol was conducted in compliance with all relevant ethical regulations. Written informed consent was obtained from all participants.

Cohort and tissue selection

Patients with an available hematoxylin and eosin (H&E) slide from pretreatment breast core biopsy tissue were identified in the MD Anderson IBC Tissue Bank. The MD Anderson IBC Tissue Registry was searched to identify which of these patients had stage III or de novo stage IV disease and received NAC followed by a mastectomy. The immune analyses of de novo stage IV disease were performed on core biopsy tissue from the primary lesion in the breast as opposed to metastatic tissue, facilitating comparisons with stage III disease. Clinicopathologic data were recorded, including age, race, stage, grade, histology, and receptor subtype. Treatment information (including use of chemotherapy, anti-HER2 targeted therapy, and endocrine therapy) and outcome data (including pathologic responses, progression-free survival (PFS), and overall survival (OS)) were also recorded. PFS was computed from the time of surgery to the time of relapse if stage III disease or clinical progression as deemed by the treating physician if stage IV disease or death, and OS from the time of surgery to time of death. Patients were categorized into responder and nonresponder groups for immune profiling (Supplementary Fig. S1). The use of the tissue and clinical data for these studies was covered under a protocol approved by the MD Anderson Institutional Review Board.

Immune profiling

Tumor-infiltrating lymphocyte assessment. From each case, an H&E slide was assessed for tumor-infiltrating lymphocytes (TIL). TILs were enumerated on H&E slides from pretreatment core biopsies according to the International TIL Working Group consensus guidelines (13). TILs were quantified by a breast cancer pathologist as a percentage of stromal area occupied by mononuclear inflammatory cells based on H&E slides.

Singlet IHC staining and quantification

IHC was performed using an automated stainer (Leica Bond Max, Leica Biosystems) staining for CD8 (Thermo, clone C8/144B, catalog no. MS-457-S, 1:20 dilution) and PD-L1 (pharmDx, clone 22C3, 1:40 dilution). Slides were stained using previously optimized conditions with appropriate positive and negative controls. Leica Bond Polymer Refine detection kit and DAB chromagen were used and counterstained with hematoxylin. Slides were scanned into an Aperio slide scanner (Aperio AT Turbo, Leica Biosystems) and analyzed with Aperio Image Toolbox software for CD8+ cell staining. Regions of invasive tumor and associated stroma were chosen for digital analysis, and quantification of cell density (cells/mm2) was performed. PD-L1 expression was scored as the percentage of positive cells with membranous or cytoplasmic staining pattern (0–100).

Multiplex IHC staining and singlet quantification

Sequential multiplex IHC was performed as previously described (14) using a myeloid panel and a lymphoid panel. Briefly, FFPE tissue sections were deparaffinized, counterstained in hematoxylin, and then the whole slide scanned into an Aperio slide scanner. After blocking with endogenous peroxidase and heat-based antigen retrieval, the slides underwent sequential iterative cycles of blocking with 5% normal goat serum/2.5% BSA/1 × PBS, primary antibody incubation, secondary horseradish conjugated antibody incubation with Histofine (mouse or rabbit) Simple Stain MAX PO (Nichirei Biosience Inc.) for 30 minutes, and then visualization with 3-aminio-9-ethylcarbazole (AEC) detection. The slides were subsequently scanned by Aperio slide scanner after which they were destained with an alcohol gradient, followed by heat-based antibody stripping and antigen retrieval. This cycle was repeated for sequential staining on a single FFPE slide.

The myeloid panel was modified from the original published protocol (14) with the following cycle order, primary antibodies, and incubation times: hematoxylin (Dako 53301, 1 minute), tryptase (AA1, Abcam, 1:20000, blocking 10 minutes, primary
30 minutes, AEC 10 minutes), CD68 (PG-M1, Abcam, 1:50, blocking 10 minutes, primary 30 minutes, AEC 10 minutes), CD8 (C8/144B, Thermo Scientific, 1:25, blocking 20 minutes, primary 30 minutes, AEC 25 minutes), CD163 (10D6, Thermo Scientific, 1:100, blocking 10 minutes, primary 30 minutes, AEC 10 minutes), HLA-DR (SPM288, Novus Biological, 1:100, blocking 10 minutes, primary 30 minutes, AEC 15 minutes), and cytokeratin (AE1/AE3 1:50, MNF116 1:50, blocking 10 minutes, primary 30 minutes, AEC 5 minutes). The lymphoid panel was modified with the following cycle order, primary antibodies, and incubation times: hematoxylin (Dako S3301, 1 minute), CD3 (SP7, Thermo Scientific, 1:150, blocking 20 minutes, primary 60 minutes, AEC 15 minutes), Foxp3 (236A/E7, eBioscience, 1:100, blocking 20 minutes, primary 60 minutes, AEC 20 minutes), CD8 (C8/144B, Thermo Scientific, 1:25, blocking 20 minutes, primary 30 minutes, AEC 25 minutes), CD20 (L26, Agilent DAKO, 1:300, blocking 20 minutes, primary 30 minutes, AEC 10 minutes), CD56 (123C3, DAKO, 1:100, blocking 10 minutes, primary 60 minutes, AEC 15 minutes), and cytokeratin (AE1/AE3 1:50, MNF116 1:50, 8 and 18 1:25, blocking 10 minutes, primary 30 minutes, AEC 5 minutes). Singlet quantification of stains was performed as described above.

DNA extraction
Sections from paraffin-embedded tissue were reviewed for pathologic diagnosis and dissected if necessary to ensure that ≥90% of the sample represented tumor. Total cellular DNA was isolated from tissue sections using the QIAamp FFPE DNA isolation kit according to the manufacturer’s protocol (Qiagen Inc.) following deparaffinization and proteinase K treatment.

T-cell receptor (TCR) sequencing and analysis
TCR beta chain CDR3 regions were sequenced by immunoseq (Adaptive Biotechnologies), with primers annealing to V and J segments, resulting in amplification of rearranged VDJ segments from each cell. Clonality and richness values were obtained through the Analyzer website. Clonality was measured as 1 – (entropy)/log2(# of productive unique sequences), with entropy taking into account clone frequency.

Cell localization and spatial analysis
Spatial localization. As previously described (14), the images acquired from the multiplex IHC staining were aligned by calculation of xy coordinates of acquired from the multiplex IHC staining were aligned by calculation of xy coordinates of fixed structures within each image and then adjustment was made through CellProfiler. Using ImageJ, these images were converted to gray scale and contrast adjusted to discriminate positive staining from background staining. To quantify the spatial proximities of cells exhibiting the queried markers, candidate nuclei in the hematoxylin IHC-stained slides were identified using the connected component analysis algorithm (15) with the MATLAB function ‘bwconncomp’ (16). Candidates whose diameters were too small or too large to be a cell (<4 μm and >16 μm, respectively) were rejected, and IHC stains of markers were overlaid over the nuclei candidates to determine which cells were positive for each marker.

Spatial G-function. The spatial G-function was used to quantify infiltration of cells of one type into another (17). This technique was used in non–small cell lung cancer and intraductal papillary mucinous neoplasms to quantify the proximities of immune cell types to cancer cells, and shown to have a significant association with OS and risk of tumor progression (18, 19). Used previously in modeling spatial statistics in ecology (20), the G-function G(r) is a nearest-neighbor distribution function that indicates the probability of a cell of type "i" having at least one cell of type "j" within a distance *r*. If we denote all cells of type i as X_i and all cells of type j as X_j, then the following equation describes the G-function mathematically:

\[ G(r) = \text{Prob}(s(x_i, x_j) \leq r) \]

where \( s(x_i, x_j) = \min(|x_i - x_j|) \) is the minimum distance between a cell x_i and cells x_j, and Prob(.) indicates the probability distribution function of the quantity within the bracket.

Consequently, this function rises sharply when cells of types "i" and "j" are clustered together, and sluggishly when the two groups of cells are further away from each other. Thus, the shape of the G-function provides a signature of the infiltration pattern. The G-function can be efficiently and compactly represented by computing (18), the area under the G-function curve (G-function AUC). The G-function AUC is computed between a distance r = 0 and r = r_{max}, as a quantitative surrogate of the amount of infiltration of one cell type into another. Various values of r_{max} between 5 and 50 μm were experimented with. The spatial analysis was performed using R’s spatstat package (21).

Statistical design
Associations between categorical measures were evaluated using the χ² or Fisher exact test as appropriate, whereas differences in continuous measures between groups were tested using the Mann–Whitney U test (if only two groups) or Kruskal–Wallis test (if more than two groups). Correlations between continuous variables were calculated using Spearman rho. Fold change in infiltration in posttreatment tissue was made between the highest and lowest quartiles of immune cell infiltration. All analyses were performed using GraphPad Prism 7. All statistical tests used an alpha value of 0.05 and were two-sided.

Results
Cohort characteristics
A total of 86 IBC patients with available pretreatment H&E-stained slides and who underwent mastectomy after NAC were identified in the IBC tumor registry and tissue bank. Baseline demographics, tumor characteristics, treatment regimens, and pathologic outcome data are shown in Table 1. Approximately 70% of cases had stage III disease with the rest having de novo stage IV disease: 90% had pure ductal histology with the remainder largely having mixed histology, and 70% of cases had grade III disease. HER2/neu-amplified (HER2 \(^{+}\)) and hormone receptor–positive, HER2/neu nonamplified (HER\(^{+}\)/HER2 \(^{-}\)) breast cancer receptor subtypes each represented 40% of the cohort population, and treatment regimens largely reflected this, with
Characterization of immunologic aspects of the TME

The IBC TME was first interrogated by presenting stage and receptor subtype among the stage III cohort (Supplementary Fig. S8G). All comparisons of receptor subtypes within stage III disease showed that HER2+ disease had significantly higher numbers of CD8+ cells compared with HR+/HER2+ disease (P = 0.003), and that TNBC had higher CD20+ cells compared with HR+/HER2+ disease (P = 0.027; Fig. 1G–H; Supplementary Fig. S6A–S6H).

PD-L1 expression at ≥1 tumor cell positivity was demonstrated in only 1 of 43 cases (Supplementary Fig. 3E), and this case displayed only 1% staining. In contrast, approximately 30% of immune cells were positive for PD-L1 staining in this cohort but with no differences by stage or receptor subtype (Fig. 1I; Supplementary Fig. S4F).

To further characterize the specificity of the T-cell infiltrate, T-cell repertoire analysis was performed. No differences in T-cell clonality (reactivity) or T-cell richness (diversity) were seen by IBC stage or receptor subtype (Fig. 1J; Supplementary Figs. S4G and S6I–S6J).

TLRs and mast cells are associated with response to neoadjuvant chemotherapy

We next asked if immune cell subsets were different between responders, defined as those experiencing pCR, and nonresponders (Fig. 2A–G; Supplementary Figs. S7A–S7J and S8A–S8M). Consistent with prior reports in non-IBC breast cancer, the presence of TILs was increased in the tumors of patients experiencing pCR (P = 0.035) with higher subsets of CD8+ (P = 0.047) and CD20+ cells (P = 0.054; Fig. 2A–C).

The immune marker most significantly associated with response was tryptase, a mast cell marker. Numbers of tryptase+ cells were significantly higher in nonresponders than in responders (P = 0.004; Fig. 2D). Representative tryptase staining in nonresponder and responder cases is shown in Supplementary Fig. S3N and S3O, respectively. This association was seen in the full cohort, in stage III cases only, and also within each receptor subtype among the stage III cohort (Supplementary Fig. S8G). All other lymphoid and myeloid markers tested as well as PD-L1 and T-cell clonality and diversity metrics were not associated with response.

To further understand what immune interactions may account for this inverse relationship between mast cells and response, spatial assessment was performed on the multiplex myeloid panel (Fig. 2E–G). Focusing on 5 μm, 10 μm, and 20 μm distances between cells, within presumed range of interaction, we found that mast cells were within close proximity to CD8+ T cells, CD163+ macrophages, and tumor cells (as assessed by cytokeratin) in nonresponders compared with responders (Fig. 2H–J; Supplementary Fig. S7I–S7J), suggesting mast cells may be exerting immunosuppressive effects by interaction with these cell types in particular.

40% receiving anti-HER2-targeted regimens in combination with chemotherapy. During the time period over which this cohort was assembled, a clinical trial of carboplatin and paclitaxel in combination with standard anthracycline- and taxane-based therapy (NCT01036087) was ongoing for patients with triple-negative breast cancer (TNBC) or HR−/HER2− disease (22), and 25% of patients in this cohort received this treatment. Approximately 40% of patients experienced a pCR, and comparison of responders and nonresponders showed that the responder cohort was enriched with stage III patients and HER2+ disease (Table 1). PFS was improved in patients experiencing a pCR in both the full cohort and when analyses were limited to patients with stage III disease (Supplementary Fig. S2A–S2B).

40% receiving anti-HER2-targeted regimens in combination with chemotherapy. During the time period over which this cohort was assembled, a clinical trial of carboplatin and paclitaxel in combination with standard anthracycline- and taxane-based therapy (NCT01036087) was ongoing for patients with triple-negative breast cancer (TNBC) or HR−/HER2− disease (22), and 25% of patients in this cohort received this treatment. Approximately 40% of patients experienced a pCR, and comparison of responders and nonresponders showed that the responder cohort was enriched with stage III patients and HER2+ disease (Table 1). PFS was improved in patients experiencing a pCR in both the full cohort and when analyses were limited to patients with stage III disease (Supplementary Fig. S2A–S2B).

40% receiving anti-HER2-targeted regimens in combination with chemotherapy. During the time period over which this cohort was assembled, a clinical trial of carboplatin and paclitaxel in combination with standard anthracycline- and taxane-based therapy (NCT01036087) was ongoing for patients with triple-negative breast cancer (TNBC) or HR−/HER2− disease (22), and 25% of patients in this cohort received this treatment. Approximately 40% of patients experienced a pCR, and comparison of responders and nonresponders showed that the responder cohort was enriched with stage III patients and HER2+ disease (Table 1). PFS was improved in patients experiencing a pCR in both the full cohort and when analyses were limited to patients with stage III disease (Supplementary Fig. S2A–S2B).
**Figure 1.**

Stage III IBC has a higher infiltrate of lymphoid cells and MHC class II presentation, whereas hormone receptor–positive disease has a lower lymphoid infiltrate. A, Quantification by TIL percentage of stromal area of primary breast tissue from stage III or de novo stage IV disease (n = 62 stage III and 24 stage IV patients). Receptor subtypes are denoted with triangle indicating hormone receptor positive, HER2/neu nonamplified (HR+/HER2–) disease, closed circle indicating HER2/neu-amplified (HER2+) disease, and open circle indicating TNBC. B, Supervised clustering by stage, then receptor status and chemotherapy response of cases that received additional IHC immune assessment (n = 37 stage III and 13 stage IV patients). TIL percentage, CD3, CD8, FoxP3, CD20, CD56, CD68, CD163, tryptase, HLA-DR, and PD-L1 expression on tumor and immune cells are shown, with red indicating higher infiltration and blue lower infiltration. C–F, Quantification of CD8+, CD56+, CD20+, and HLA-DR+ cells by stage (n = 56 stage III and 13 stage IV, 18 stage III and 13 stage IV, 19 stage III and 13 stage IV, 35 stage III and 13 stage IV, respectively). G and H, Quantification of CD8+ and CD20+ cells by tumor receptor status (n = 8 TNBC, 14 HER2+, 9 HR+/HER2– for CD8+, 4 TNBC, 6 HER2+, 9 HR+/HER2– for CD20+) and by receptor subtype (n = 8 TNBC, 14 HER2+, 9 HR+/HER2–). I, Bar graph representing percentage of cases with PD-L1+ tumors and immune cells (n = 43). J, TCR clonality by stage (n = 23 stage III and 9 stage IV). A, C–H, J, Bar heights indicate median values, and interquartile ranges are presented in addition to individual data points. All comparisons were made using two-sided Mann–Whitney U test (if two groups) or Kruskal–Wallis test with adjustment for multiple testing (if more than two groups).
Mast cells are the strongest immune predictor of poor response to chemotherapy, with higher TILs seen in responders than in nonresponders. A–D, TIL percentage and densities of CD8⁺, CD20⁺, and tryptase⁺ cells by response, with response defined as achieving a pathologic complete response (pCR) or not (non-pCR; n = 52 non-pCR and 34 pCR, 33 non-pCR and 16 pCR, 24 non-pCR and 7 pCR, 32 non-pCR and 17 pCR, respectively). Closed circle indicates stage III cases, and open circle stage IV cases. E, Representative multiplex IHC image of a non-pCR and pCR case with nuclei (blue), CD68 (purple), CD163 (turquoise), tryptase (yellow), HLA-DR (green), CD8 (red), and cytokeratin (orange) stains depicted. F and G, Depiction of methodology for spatial analyses performed. Probabilities of a cell of interest being within a certain radius to another cell of interest were computed and area under the curve (AUC) calculated, with higher AUC indicating closer proximity. H, Non-pCR cases (n = 15) demonstrated higher AUC of mast cells to CD8⁺ T cells than pCR cases (n = 12). I, Representative non-pCR and pCR cases showing distance between CD8⁺ T-cell (red) and tryptase⁺ mast cell (yellow). J, Comparisons by response of AUC between mast cells and CD8⁺, CD163⁺, and cytokeratin⁺ (tumor) cells (n = 15 non-pCR and 12 pCR, 18 non-pCR and 14 pCR, 21 non-pCR and 14 pCR, respectively). A–D, J, Bar heights indicate median values, and interquartile ranges are presented in addition to individual datapoints. Comparisons were made using two-sided Mann–Whitney U test.

Figure 2.
Macrophages and MHC class II molecules are associated with improved PFS.

On a subset of cases with available posttreatment tissue from their surgical specimen (n = 27), immune profiling was performed to characterize immune infiltrates in residual tumor as well as areas of treatment effect and to study the change in immune cells with therapy (Fig. 3A–F, Supplementary Figs. S9A–S9O and S10A–S10I). Pathologic response, stage, receptor subtype, and treatment characteristics of this cohort are provided in Supplementary Table S1. The cohort included tissue retrieved from the previous tumor bed in 6 patients who experienced a pCR and residual tumor in 21 patients who did not experience a pCR. Analysis of posttreatment tissue showed that myeloid infiltrates including macrophages, assessed by CD163 (P = 0.004) and CD68 (P = 0.004), as well as HLA-DR (P = 0.011), were present in significantly higher densities in the prior tumor bed...
of cases that achieved a pCR compared with in the residual tumor of non-pCR cases (Fig. 3A–C). In addition, higher infiltration of these cell types was associated with improved PFS (P = 0.062, 0.156, and 0.012 for CD163, CD68, and HLA-DR, respectively) and a trend in improved OS among the non-pCR cases (Fig. 3D–F; Supplementary Fig. S10A–S10C). FoxP3+ and CD56+ cells were also elevated in pCR mastectomy tissue, whereas lower numbers of FoxP3+ cells were associated with trend in improved OS among non-pCR cases (Supplementary Figs. S9C, S9E, and S10G).

When looking at fold change from pretreatment biopsy specimens to mastectomy specimens (Fig. 3G), there was a significant decrease in CD8+ cells (P < 0.001) as well as tryptase+ cell density (P = 0.012) with treatment in patients who experienced a pCR. Non-pCR cases also demonstrated a significant decrease in tryptase+ cell density as well as in CD56, HLA-DR, and CD163 expression (P = 0.021, 0.043, 0.020, and 0.020, respectively). Fold change by treatment type is shown in Supplementary Fig. S11A, though it is difficult to draw conclusions on differential changes by therapy given the limited size of the cohort and that the pCR cases consisted of 5 patients who received anti-HER2–targeted therapy and 1 who received an EGFR inhibitor along with chemotherapy.

Treatment induces higher clonality as well as higher T-cell diversity in responders

T-cell repertoire analysis using TCR sequencing overall showed a significant change in repertoire with very low Morisita overlap values in both pCR and non-pCR cases (Supplementary Fig. S11B). Responders showed an increase in both clonality and diversity (richness) compared with nonresponder cases (P = 0.003 and 0.032, respectively; Fig. 3H and I).

Discussion

This study provides a large and comprehensive characterization of the IBC tumor immune microenvironment that has been performed using reliable methodology such as IHC to quantify immune cell subsets and determine their relationship to response. Mast cell infiltration, as identified by tryptase staining, was significantly associated with poor response to neoadjuvant chemotherapy in all stage and receptor subtypes of IBC and presents a possible therapeutic target.

IBC subtypes, we explored potential immune interactions contributing to treatment resistance by analysis of spatial relationships with a multiplex IHC platform. By focusing on distances up to 20 μm (33), we found that mast cells were within range for direct or paracrine interactions with CD8+ T cells, the primary effectors of antitumor responses, as well as CD163+ macrophages and tumor cells. Mast cells may therefore at least partially be exerting their inhibitory effect in IBC through suppressing CD8+ T cells, enhancing immunosuppressive CD163+ macrophages, and directly promoting tumor cell growth. Prior studies support such interactions (34–39). Though this work will need to be validated with mechanistic studies, these data suggest mast cells may be a therapeutic target to enhance responses to NAC, and already existent agents such as mast cell stabilizers and c-Kit inhibitors make this readily translatable. In addition, these findings give further support that inflammation in “inflammatory” breast cancer extends beyond clinical appearance to underlying pathophysiology.

This study also demonstrated known lymphoid biomarkers of response to NAC as seen in non-IBC, including TILs (40), CD8+ T-cell infiltrate (41), and CD20+ B-cell infiltrate (42, 43), though notably these lymphoid infiltrates were less significantly associated with response compared with mast cell infiltration. Differences were noted in density of these lymphoid infiltrates by stage and by receptor status, with stage IV disease and hormone receptor–positive disease having a “colder” TME, therefore perhaps less immunogenic tumors than other subsets, potentially accounting for differential responses to therapy in these IBC subsets. Although lower TIL and specific lymphocyte subsets have previously been seen in matched primary metastases compared with primary breast cancer sites, this current study is unique in comprehensively characterizing primary breast tissue of de novo metastatic disease and showed that a less inflamed TME is seen at the site of the primary tumor and likely precedes development of distant metastases. PD-L1 positivity (≥1%) was seen on tumor cells in only 1 of 43 pretreatment cases assessed and on immune cells in ~30% of cases. Low PD-L1 positivity, in particular tumor cell positivity, is lower than seen in non-IBC even with the same antibody clone, suggesting this is not a primary mechanism for immune evasion in IBC (7, 44–49). Consistent with this, neither tumor nor immune cell PD-L1 positivity was correlated to response in this cohort. This rate of PD-L1 expression is consistent with prior RNA expression–based studies in IBC (11), and our IHC methodology additionally provides an accurate quantification of the number of cells with positive PD-L1 expression and further subdivides into tumor versus immune cell positivity. Unlike the prior report, our finding that PD-L1 is not predictive of pCR may reflect a more accurate characterization of PD-L1 expression though limited sample size may also be contributing.

Posttreatment tissue assessment revealed greater macrophage infiltration and HLA-DR staining in the primary tumor bed of patients experiencing a complete response to NAC as well as in residual tissue of non-pCR cases that had improved PFS. Interpretation of these findings is confounded by the overrepresentation of anti-HER2– and anti-EGFR–targeted treatments combined with NAC in the responders for the posttreatment cases available. Therefore, this may reflect that monoclonal antibody–treated patients demonstrate evidence of antibody-dependent phagocytosis, in which macrophages are critical effectors, and highlight
the importance of this mechanism in addition to NK cells in mediating improved outcomes (50, 51). As expected, higher NK cell infiltration was also seen with therapy in responders. In this limited cohort, no significant relationship was seen between lymphoid infiltration in residual tumor and PFS outcome, though greater numbers of FoxP3+ cells were seen in pCR compared with non-pCR cases, perhaps again reflective of a trastuzumab treatment effect, as has been previously demonstrated (52); among the non-pCR cases, lower FoxP3+ cell infiltration was associated with improved OS as previously reported in non-IBC (53). Analysis of change in T-cell repertoire dynamics showed that response was associated with an increase in clonality but also an increase in diversity metrics. This suggests that NAC (and again in this case monoclonal antibody and specifically HER2 targeted therapy) led to an increase in overall tumor reactive T cells as well as an increase in specific expanded T-cell clones accounting for increase in clonality. Similar to what was seen in pretreatment tissue, our cases were largely PD-L1-negative on tumor cells and positive in approximately one third of cases on immune cells, neither with correlation to survival outcome. Another study using tissue microarrays of 68 mastectomy IBC cases diagnosed prior to 2004 found that 37% of non-pCR cases were positive for PD-L1 on tumor cells and that this predicted worse survival (54); differences in the patient population (earlier stage patients and unknown neoadjuvant treatment characteristics in the other cohort), PD-L1 antibody clone used, and a limited sample size in our posttreatment cohort limit meaningful comparisons between these two studies. When comparing pCR and non-pCR cases, differences between cases in the area occupied by tumor cells in the residual tumor bed of non-pCR cases (vs. pCR cases for which there are no tumor cells present) may have added bias to our posttreatment results. However, the interspersed nature of IBC infiltration into the tissue made this difficult to account for. Nevertheless, the presence of consistent findings in our study between the pCR versus non-pCR tumor bed analysis and the survival analysis among non-pCR cases lends support for the accuracy and clinical relevance of our findings regarding higher macrophage infiltration and HLA-DR+ cells in posttreatment tissue of responders. Our results will need to be validated in larger data sets with more homogeneous populations by stage, receptor status, and associated therapies.

The primary limitation of this study is the relatively small size of the cohort and heterogeneity with respect to stage, receptor status, and associated differences in neoadjuvant treatments given. These mixed classes and treatment statuses therefore limit reliability of pooling data for pre- and posttreatment analyses. However, given the relative rarity of IBC and the comprehensive assessment of primary breast tissue from de novo IBC, the work presented here provides thorough immune characterization of IBC. To ensure accurate and robust conclusions from these data, all analyses were performed for the full cohort as well as within stage and receptor subsets when possible to determine both generalizable and subset-specific findings. These will need to be validated in additional IBC cohorts as well as compared with matched non-IBC to better understand the unique pathophysiology of IBC. In addition, with respect to our findings on mast cell infiltration predicting poor response to chemotherapy, mechanistic studies in addition to functional and activation profiling of the immune subsets (such as mast cells and CD8+ T cells) are needed to further validate our findings and identify optimal modalities of therapeutically targeting IBC tumors.

In summary, this study provides a comprehensive immune profiling of IBC from which future studies can build. We showed expected biomarkers of response to NAC such as TILs and CD8+ T-cell infiltration, which have a known role across solid tumors in shaping response to therapy. We additionally identified mast cells as significantly associated with poor responses to therapy and a potential therapeutic target, highlighting the role of nonlymphoid subsets in IBC and perhaps even more generally the breast cancer TME. Together, these analyses provide an integrated profile of IBC to inform future design of IBC-specific clinical trials.

Disclosure of Potential Conflicts of Interest
V. Gopalakrishnan has received honoraria from the speakers bureau of the Kansas Society of Clinical Oncology and Excel CME, has ownership interest (including stock, patents, etc.) in UI/MD Anderson Cancer Center, and is a consultant/advisory board member for MicrobiomeDX and Expert-Connect. M.T. Tetzlaff is a consultant/advisory board member for Novartis, LLC, Myriad Genetics, and Seattle Genetics. L.M. Coussens reports receiving commercial research grants from Acerta Pharma, LLC, Desiprhea Pharmaceuticals, LLC, Roche Glycart AG, and Syndax Pharmaceuticals, Inc., reports receiving other commercial research support from Plexxikon Inc., Pharmacies, Inc., Acerta Pharma, LLC, Desiprhea Pharmaceuticals, LLC, Genentech, Inc., Roche Glycart AG, Cell Signaling Technology, and Nano-String Technologies, Inc., is a consultant/advisory board member for Pharcymecles Inc., Inc. steering committee for PCG-1137-CA (NCT02419648), Syndax Pharmaceuticals, Inc. External Advisory Board, Cancer Research Institute (CRI), The V Foundation for Cancer Research, Cancer Research United Kingdom (CRUK) Early Detection (EDs) Research Committee, Starr Cancer Consortium, NIH/NCI-Frederick National Laboratory Advisory Committee (FNLLAC), Cell Signaling Technology; Carisma Therapeutics Inc. Scientific Advisory Board, Verseau Therapeutics, Inc, Scientific Advisory Board, Zymergen, Inc. Scientific Advisory Board, (P30) Melvin and Bren Simon Cancer Center, Indiana University, (P30) Koch Institute for Integrated Cancer Research, Massachusetts Institute of Technology, (P30) Salk Institute Cancer Center, Bloomberg-Kimmel Institute for Cancer Immunotherapy, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, and Dana Farber Cancer Center Breast SPORE. A. Rao is a member of Voxel Analytics, reports receiving other commercial research support from Agilent Technologies, and is a consultant/advisory board member for Deoxylitics. N.T. Ueno reports receiving a commercial research grant from Amgen and Celgene. J.A. Wargo has received honoraria from the speakers bureau of Dava Oncology, illumina, and PHiE and is a consultant/advisory board member for Bristol-Myers Squibb, Novartis, Roche, Genentech, AstraZeneca, and Merck. E.A. Mittendorf is a consultant/advisory board member for Merck, Sellas LifeSciences, AstraZeneca, TapImmune, Peregine Pharmaceuticals, and Genentech. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Reddy, A. Reuben, C.W. Hudgens, M.T. Tetzlaff, K. Wani, A. Wood, A. Rao, S. Krishnamurthy, J.A. Wargo, E.A. Mittendorf
Writing, review, and/or revision of the manuscript: S.M. Reddy, A. Reuben, S. Barua, L. Wang, V. Gopalakrishnan, M.T. Tetzlaff, A. Rao, J.A. Wargo, N.T. Ueno, S. Krishnamurthy, J.A. Wargo, E.A. Mittendorf
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M. Reddy, H. Jiang, Y. He, L. Villareal, A. Rao, N.T. Ueno, E.A. Mittendorf
Reddy et al.

Study supervision: S.M. Reddy, S. Krishnamurthy, E.A. Mittendorf
Other (wrote software modules for cell localization and spatial infiltration analysis): S. Barua
Other (spatial analysis, statistics): A. Rao

Acknowledgments
This research was funded by the Breast Cancer Research Foundation/Conquer Cancer Foundation Young Investigator Award (to S.M. Reddy), Breast Cancer Research Foundation (to N.T. Ueno), Morgan Welch Inflammatory Breast Cancer Research Program and Clinic, State of Texas Rare and Aggressive Breast Cancer Research Program Grant, and the MD Anderson Cancer Center Support grant (NCI #CA16672). S.M. Reddy received support from NIH T32 Training Grant T32 CA093666. A. Reuben received support from the Kimberly Clark Foundation Award for Scientific Achievement provided by MD Anderson’s Odyssey Fellowship Program. A. Rao received support from the CCSG Bioinformatics Shared Resource P30 CA016672, an Institutional Research Grant from The University of Texas MD Anderson Cancer Center, CPRIT RP170719, CPRIT RP150578, NCI R01CA214955-01A1, a Career Development Award from the MD Anderson Brain Tumor SPORE, a gift from Agilent technologies, and a Research Scholar Grant from the American Cancer Society (RSG-16-005-01). E.A. Mittendorf received support from the Pink Ribbons Project.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 5, 2018; revised December 18, 2018; accepted April 22, 2019, published first May 1, 2019.

References
Mast Cells and Poor Response in Inflammatory Breast Cancer

Cancer Immunology Research

Poor Response to Neoadjuvant Chemotherapy Correlates with Mast Cell Infiltration in Inflammatory Breast Cancer


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-18-0619

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2019/05/01/2326-6066.CIR-18-0619.DC1

Cited articles
This article cites 50 articles, 5 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/7/6/1025.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerimmunolres.aacrjournals.org/content/7/6/1025.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link:
http://cancerimmunolres.aacrjournals.org/content/7/6/1025.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.