Interferon Signaling Is Diminished with Age and Is Associated with Immune Checkpoint Blockade Efficacy in Triple-Negative Breast Cancer

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ABSTRACT

Immune checkpoint blockade (ICB) therapy, which targets T cell–inhibitory receptors, has revolutionized cancer treatment. Among the breast cancer subtypes, evaluation of ICB has been of greatest interest in triple-negative breast cancer (TNBC) due to its immunogenicity, as evidenced by the presence of tumor-infiltrating lymphocytes and elevated PD-L1 expression relative to other subtypes. TNBC incidence is equally distributed across the age spectrum, affecting 10% to 15% of women in all age groups. Here we report that increased immune dysfunction with age limits ICB efficacy in aged TNBC-bearing mice. The tumor microenvironment in both aged mice and patients with TNBC shows decreased IFN signaling and antigen presentation, suggesting failed innate immune activation with age. Triggering innate immune priming with a STING agonist restored response to ICB in aged mice. Our data implicate age-related immune dysfunction as a mechanism of ICB resistance in mice and suggest potential prognostic utility of assessing IFN-related genes in patients with TNBC receiving ICB therapy.

SIGNIFICANCE: These data demonstrate for the first time that age determines the T cell–inflamed phenotype in TNBC and affects response to ICB in mice. Evaluating IFN-related genes from tumor genomic data may aid identification of patients for whom combination therapy including an IFN pathway activator with ICB may be required.

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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INTRODUCTION

Recent clinical successes using immune checkpoint blockade (ICB) have led to FDA approval of antibody-based therapeutics that target T cell-inhibitory receptors, including CTLA4, PD-1, and PD-L1. These therapies have revolutionized cancer treatment, particularly for melanoma and non-small cell lung cancer (NSCLC; refs. 1–5). Focus has recently shifted to applying ICB to other tumor types including breast cancer. Breast cancers that do not express hormone receptors (estrogen receptor, progesterone receptor) and lack amplification of HER2 are classified as triple-negative breast cancer (TNBC), which lacks targeted therapy and is the most aggressive subtype of the disease (6). Evaluation of ICB has been of greatest interest in TNBC due to higher numbers of tumor-infiltrating lymphocytes (TIL) and elevated PD-L1 expression, which correlate with response to ICB, relative to other breast cancer subtypes (7–10). ICB monotherapy has proven effective for approximately 10% of patients with TNBC (11, 12). The IMpassion130 trial recently reported that patients with metastatic TNBC whose tumors express PD-L1 significantly benefited from anti-PD-L1 (atezolizumab) when administered with the chemotherapy nab-paclitaxel (13), leading to recent FDA approval of atezolizumab as first-line therapy for metastatic TNBC. ICB represents an improvement over standard therapeutic strategies for TNBC; however, the reasons that ICB therapy has had selected efficacy for patients with TNBC (14) are unknown.

Age is associated with increasing immune dysfunction that includes significant changes to both innate and adaptive immunity (15–17). Most notably, hematopoiesis is skewed toward myeloid production whereas lymphopoiesis retracts with age. Some of the age-related changes specific to peripheral T-cell populations include fewer naïve T cells, increased numbers of terminally differentiated memory T cells, reduced expression of the costimulatory molecule CD28, and increased exhaustion, evidenced by PD-1 expression (18). Antigen-presenting cells including dendritic cells (DC) and macrophages also have decreased activity, whereas immunosuppressive populations, such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), have increased activity with age in patients with cancer (19). Although most aspects of innate and adaptive immunity are different between young and elderly individuals, the clinical implications of these age-related changes are unknown.

Age-related immune dysfunction could therefore present particular challenges to efficacy of immunotherapy in breast cancer.
cancer, as more than 50% of patients are older than 60 years of age at diagnosis (20). However, most clinical studies either exclude or under-enroll older patients (21) and do not include tissue-based analyses that can help predict for efficacy. Given the limited number of older patients enrolled on clinical trials evaluating ICB in TNBC, it is not known whether age affects response to therapy. We therefore initiated a study in murine models of TNBC to understand whether age-related immune dysfunction influences ICB efficacy in TNBC.

RESULTS

Age-Dependent Effects on Peripheral Immunity, TNBC Progression, and Tumor Immune Microenvironment

To identify murine models correlating with age-associated immune changes seen in humans, we first assessed age-associated differences in peripheral immune cells from young (8–10 weeks) and aged (>12 months) BALB/c and FVB mice. Lymphocytes comprised the majority of circulating white blood cells (WBC) in both strains of mice, irrespective of age (Fig. 1A and B). However, total lymphocyte counts, including both CD4+ and CD8+ T cells, were significantly reduced in both strains of aged mice compared with young mice (Fig. 1A–D). The circulating myeloid cell compartment was significantly expanded in both strains with age (Fig. 1A and B). Within the myeloid compartment, neutrophil counts were elevated in both strains whereas monocyte counts were altered in a strain-specific manner (Fig 1A and B).

Given that aged mice reflected the myeloid bias and decreased lymphocyte production observed in humans with age (22–24), we proceeded to test whether age also affects tumor progression and development of the tumor microenvironment (TME). To do so, we used 4T1 and Met1 transplantable orthotopic models of TNBC in BALB/c and FVB mice, respectively.

In the BALB/c mice, 4T1 mammary carcinoma onset and incidence (100%) were similar between young and aged cohorts (Fig. 1E). Tumor growth kinetics were modestly increased in the aged cohort during the early stages of tumor development, but ultimately were similar in both young and aged cohorts by late-stage disease (Fig. 1E; Supplementary Fig. S1A). In the FVB mice, Met1 tumor onset and incidence (100%) were the same in both cohorts; however, tumor growth rates were significantly reduced in the aged mice (Fig. 1F; Supplementary Fig. S1B). Testing various other mammary carcinoma cells revealed similar strain-specific differences; 4T07 and 67NR tumors grew similarly in young and aged BALB/c mice and McNeuA tumors grew more slowly in aged than in young FVB mice (Supplementary Fig. S1C and S1D), suggesting that age-related host physiology may affect tumor growth.

We next assessed tumor-related changes to the peripheral immune system with age. Relative to cancer-free animals, circulating lymphocytes were reduced in young tumor-bearing mice, and this reduction was even more enhanced in aged tumor-bearing mice (Fig. 1G and H; Supplementary Fig. S1E). Young and aged tumor-bearing BALB/c and FVB mice exhibited neutrophilia, which was more pronounced in the aged cohort than in the young cohort (Fig. 1I and J; Supplementary Fig. S1F). In young BALB/c mice, we observed a reduction in circulating monocytes with tumorigenesis; however, in aged BALB/c mice, which already had lower monocyte counts, these levels were unchanged with tumorigenesis (Fig. 1K; Supplementary Fig. S1G). In the FVB mice, neither age nor tumorigenesis resulted in altered monocyte levels (Fig. 1L).

We next examined the TME by IHC. There were no significant age-dependent differences in the percentage of Ki67+ proliferating cells or myofibroblasts (α-SMA) in 4T1 or Met1 tumors (Supplementary Fig. S1H and S1I). Macrophage (F4/80+) infiltration was significantly reduced in 4T1 tumors from aged BALB/c mice relative to young, but unchanged in Met1 tumors from FVB mice (Supplementary Fig. S1H and S1I). We observed an increase in the overall percentage of intratumoral neutrophils (MPO+) in 4T1 tumors compared with Met1 tumors (Supplementary Fig. S1H and S1I), but no difference when comparing young and aged mice in the respective models. All mice also exhibited tumor-dependent neutrophilia in the blood (Supplementary Fig. S1H and S1I). Intratumoral infiltration of CD3+ and CD8+ T cells was also unchanged with age in both strains of mice (Fig. 1M–P).

Numbers of Tregs were significantly reduced in 4T1 tumors from aged mice and increased in the aged Met1 tumor-bearing mice (Fig. 1M–P); nevertheless, these changes were not sufficient to significantly alter the intratumoral CD8+ Treg ratio in either strain (Fig. 1M–P).

Differential Responses to ICB Therapy with Age

The age- and tumor-dependent changes to peripheral immunity and the TME that we observed raised the question of whether age influences response to immunotherapy, particularly checkpoint blockade. We first verified that the 4T1 and Met1 cells express PD-L1 and significantly increased cell-surface PD-L1 and MHC I expression in response to IFNγ

Figure 1. Age-dependent effects on hematopoiesis, TNBC tumor growth, and TILs. Hemavet analysis of peripheral blood from young and aged cancer-free (CF) BALB/c (A) and FVB (B) mice. Values represent percentage of total white blood cells (n = 5 mice/cohort). Flow cytometric analysis of peripheral blood CD4+ and CD8+ T lymphocyte populations as a percentage of viable cells in young and aged cancer-free BALB/c (C) or FVB (D) mice (n = 5 mice/cohort). Growth kinetics of 4T1 tumors in young and aged BALB/c mice (n = 5 mice/cohort; E) and Met1 tumors in young and aged FVB mice (n = 5 mice/cohort; F). Representative of two independent experiments. Hemavet analysis of lymphocytes in the BALB/c (G) and FVB (H) as a percentage of total WBCs in cancer-free and tumor-bearing mice (n = 4–5 BALB/c mice/cohort; n = 10 FVB mice/cohort). Hemavet analysis of neutrophils in the BALB/c (I) and FVB (J) as a percentage of total WBCs in cancer-free and tumor-bearing mice (n = 4–5 BALB/c mice/cohort; n = 10 FVB mice/cohort). Hemavet analysis of monocytes in the BALB/c (K) and FVB (L) as a percentage of total WBCs in cancer-free and tumor-bearing mice (n = 4–5 BALB/c mice/cohort; n = 10 FVB mice/cohort). M, Representative immunofluorescence images of 4T1 tumors from young and aged BALB/c mice stained for CD3 (green), FOXP3 (red), and DAPI (blue). N, Average number of CD3+, CD25/FOXP3, and CD8+ T cells and ratio of CD8+ T cells:Tregs per 4T1 tumor from young and aged BALB/c mice; n = 11 tumors/cohort from two independent experiments—each data point represents average count from 4–5 fields/tumor. O, Representative immunofluorescence images of Met1 tumors from young and aged FVB mice stained for CD3 (green), FOXP3 (red), and DAPI (blue). P, Average number of CD3+, CD25/FOXP3, and CD8+ T cells and ratio of CD8+ T cells:Tregs per Met1 tumor from young and aged FVB mice; n = 5–6 tumors/cohort–each data point represents average count from 4–5 fields/tumor. Two-sided unpaired t test (N and P) with Welch correction (A–L). Error bars, SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
(Supplementary Fig. S2A–S2D), indicating their potential to be recognized by immune cells (CD8+ T cells).

We generated orthotopic 4T1 and Met1 TNBC in cohorts of young (8-10 weeks) and aged (>12 months) mice. Once tumors reached approximately 5 mm³ in volume, mice were randomized into treatment cohorts and administered four doses of anti–PD-L1, anti-CTLA4, or isotype antibody control (Supplementary Fig. S2E–S2G).

In four independent experiments in the BALB/c mouse, only the young cohort exhibited significantly decreased 4T1 tumor growth after either anti–PD-L1 or anti-CTLA4 treatment compared with isotype controls; neither treatment was effective in the aged mice (Fig. 2A and B; Supplementary Fig. S2H). In separate experiments to measure survival, anti–PD-L1 and anti-CTLA4 each improved overall survival of young mice relative to the young isotype controls; however, neither treatment affected survival of the aged mice (Fig. 2C; Supplementary Fig. S2I).

We had earlier observed that Met1 tumor growth was attenuated in aged mice relative to young mice (Fig. 1F); therefore, we did not directly compare young and aged FVB cohorts to one another and instead made comparisons only within each age cohort. In four independent experiments, anti–PD-L1 and anti-CTLA4 treatment significantly slowed the rate of Met1 tumor growth in young mice, although no complete regressions were observed (Fig. 2D and E; Supplementary Fig. S2J). In the aged mice from these experiments, anti–PD-L1 treatment resulted in a slight reduction in tumor growth, although this was not statistically significant, and the aged mice did not respond to anti-CTLA4 (Fig. 2F and G; Supplementary Fig. S2K). Aged FVB mice gained significant overall survival benefit from anti–PD-L1 but not anti-CTLA4 therapy (Fig. 2I; Supplementary Fig. S2K).

To determine whether adaptive immunity is necessary for response to ICB, we first orthotopically injected 4T1 or Met1 TNBC cells into young and aged nude mice, which lack mature T cells, and administered ICB according to our protocol (Supplementary Fig. S2E). Aged nude mice bearing 4T1 tumors did not respond to anti–PD-L1 treatment (Fig. 2J; Supplementary Fig. S2L). The young nude mice showed 4T1 tumors did not respond to anti–PD-L1 treatment only at the experimental endpoint; however, this reduction was modest relative to immunocompetent mice (Supplementary Fig. S2M–S2O). By neutralizing CD8+ T cells were necessary for response to anti–PD-L1 and anti-CTLA4 were manifested.

We first analyzed age- and treatment-associated changes in T-cell phenotypes in peripheral blood cells by flow cytometry (Supplementary Fig. S3A). We focused on analysis of naive T cells (no prior antigen exposure), memory phenotypes (prior exposure to antigen), and cell-surface checkpoint protein expression (indicating active immune responses as well as loss of both proliferative potential and effector function). Age alone was associated with approximately 3-fold increased numbers of circulating effector memory (EM) CD8+ cells (CD45+/CD3+/CD8+/CD44+/CD62L−) and approximately 2-fold increases in CD8+/PD-1+ T cells relative to young isotype-treated cohorts (Fig. 3A; Supplementary Fig. S3B).

Anti-CTLA4 treatment resulted in approximately 2-fold increased numbers of circulating naive CD8+ T cells (CD45+/CD3+/CD8+/CD44−/CD62L−) and approximately 2-fold increases in PD-1+/CD8+ cells in the young cohort, but not in the aged cohort (Fig. 3A; Supplementary Fig. S3B).

We similarly analyzed spleen cells. Isotype-treated aged mice had approximately 4-fold higher numbers of splenic CD8+/PD-1−/TIM3− T cells, approximately 7-fold higher numbers of CD8+ EM cells, and approximately 4-fold higher numbers of PD-1+ EM cells than young mice (Fig. 3B; Supplementary Fig. S3C). In response to ICB, the numbers of PD-1−/naive T cells (CD45+/CD3+/CD8+/CD44−/CD62L−/PD-1−) were significantly reduced only in spleens of young mice (Fig. 3B; Supplementary Fig. S3C). In response to anti–PD-L1, the numbers of CD3+/PD-1−/TIM3− cells were enhanced approximately 2.5-fold in the aged mice but not in the young mice (Fig. 3B; Supplementary Fig. S3C).

Collectively, our analysis of blood and spleen lymphocytes indicated that age is associated with enhanced CD8+ T-cell EM and exhaustion phenotypes, as previously reported in tumor-free aging models of immune dysfunction (25). Importantly, peripheral T-cell phenotypes with ICB treatment were reflective of active immune response in young but not aged mice.

Given these results, we tested the proliferation capacity of CD8+ T cells from spleen and lymph nodes of young and aged mice. CD8+ T-cell proliferation in response to CD3/CD28 stimulation ex vivo was not significantly different between cohorts (Fig. 3C). However, the number of checkpoint proteins (PD-1, CTLA4, LAG3, and/or TIM3) expressed per cell was significantly increased on CD8+ T cells from aged mice, but not young mice, after stimulation (Fig. 3D and E). Among the CD8+ populations from aged mice, PD-1+/TIM3−, PD-1+/CTLA4−, and PD-1+/CTLA4+/TIM3− cells underwent the greatest expansion in response to stimulation (Fig. 3F). The PD-1+/CTLA4+ CD8+ cells from young mice expanded most significantly following stimulation (Fig. 3F). Collectively, these results demonstrated that CD8+ T cells from aged mice are capable of proliferation but also concurrently increase expression of checkpoint proteins more so than T cells from young mice in response to stimulation.
Figure 2. Differential responses to ICB therapy with age. Average growth (A) and endpoint mass (B) of 4T1 tumors from young and aged BALB/c mice treated with either 200 μg isotype control antibody, 200 μg anti–PD-L1, or 200 μg anti–CTLA4 on days 7, 10, 13, and 16 (arrows) postinjection (n = 4–7 mice/cohort); representative of four independent experiments. C, Kaplan–Meier survival analysis of young and aged BALB/c mice bearing 4T1 tumors that were treated with either 200 μg isotype control, 200 μg anti–PD-L1, or 200 μg anti–CTLA4 on days 7, 10, 13, and 16 (arrows) postinjection (n = 4–7 mice/cohort from 1 experiment). Average growth (D) and endpoint mass (E) of Met1 tumors from young FVB mice treated with either 200 μg isotype control, 200 μg anti–PD-L1, or 200 μg anti–CTLA4 on days 7, 10, 13, and 16 (arrows) postinjection (n = 7–8 mice/cohort) representative of four independent experiments. Average growth (F) and endpoint mass (G) of Met1 tumors from aged FVB mice treated with either 200 μg isotype control, 200 μg anti–PD-L1, or 200 μg anti–CTLA4 on days 7, 10, 13, and 16 (arrows) postinjection (n = 7 mice/cohort); representative of four independent experiments. H, Kaplan–Meier survival analysis of young FVB mice bearing Met1 tumors that were treated with either 200 μg isotype control, 200 μg anti–PD-L1, or 200 μg anti–CTLA4 on days 7, 10, 13, and 16 (arrows) postinjection (n = 7–8 mice/cohort); representative of four independent experiments. J, Average growth of 4T1 tumors in young and aged nude mice treated with either 200 μg isotype control or 200 μg anti–PD-L1 on days 6, 9, 12, and 15 postinjection (n = 5–10 mice/cohort representing 1 experiment). K, Growth kinetics of 4T1 tumors in young BALB/c mice treated with 100 μg isotype control, 100 μg anti–PD-L1, or 100 μg anti–PD-L1 + 100 μg anti–CD8 according to the dosing regimen in Supplementary Fig. S3E (n = 5–6 mice/cohort representing 1 experiment). Two-way ANOVA followed by the Sidak multiple comparison test (A and J), one-way ANOVA followed by multiple comparison test (B, D, E, G, H, K, and L), and log-rank (Mantel–Cox) test (C, F, I). Error bars, SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
**Figure 3.** Age- and ICB-dependent differences in TILs and exhaustion phenotypes. Heat maps representing fold changes in numbers of cells expressing indicated checkpoint proteins in peripheral blood lymphocytes (A) and splenocytes (B) associated with age and ICB therapy; numbers were analyzed by flow cytometry and are represented relative to the young isotype-treated mice (n = 4–5 mice/cohort). C, Percentage of CD8+ T cells isolated from spleen and lymph nodes of indicated mice that proliferated in vitro 72 hours after stimulation with anti-CD3/CD28 beads (2 T cells:1 bead); n = 3 mice per condition with three technical replicates per mouse. Number of immune checkpoint proteins (PD-1, CTLA4, TIM3, LAG3) expressed per stimulated and unstimulated CD8+ T cells from young (B) and aged (E) mice (n = 3 mice per condition with three technical replicates). D, Repertoire of CD8+ T-cell exhaustion phenotypes (by indicated checkpoint protein profile) resulting from unstimulated and stimulated CD8+ T-cell cultures (n = 3 mice per condition with three technical replicates for stimulated CD8+ T cells). (continued on following page)
Figure 3. (Continued) G, Representative images of 4T1 tumor sections stained for CD3 (green), FOXP3 (red), and nuclei (blue). Scale bar, 100 μm. Average number of CD3+ lymphocytes (H) and CD3+/FOXP3+ Tregs (I; n = 5–11 4T1 tumors) and 4–5 fields/tumor for each cohort representing two independent experiments. J, Representative images of 4T1 tumor sections stained for CD8 (red) and nuclei stained with hematoxylin (blue). Scale bar, 100 μm. K, Average number of CD8+ lymphocytes (n = 5–10 tumors) and 4–5 fields/tumor for each cohort from two independent experiments. L, Ratio of intratumoral CD8+ lymphocytes to Tregs; n = 5–9 tumors analyzed from two independent experiments. M, CD8+ T-cell localization in 4T1 tumors as blindly assessed by two independent investigators. S, stromally restricted; T, intratumoral; P, peripherally restricted; localization stratified into 5 groups: “cold” (no CD8+ cells), “restricted” (P or S only), “minor infiltration” (S or P > T), “equivalent infiltration” (T = S), and “hot” (T > S or P); n = 5–10 tumors analyzed per cohort from two independent experiments. N, Heat map representing fold changes in absolute numbers of cells expressing indicated checkpoint proteins in TILs associated with age and treatment; values were determined using flow cytometry and represented relative to the young isotype-treated mice; (n = 4–5 mice/cohort). Two-sided unpaired Student t-test (C–F). Error bars, SEM. *P < 0.05, **P < 0.01, ***P < 0.001. CM, central memory (CD45+/CD3+/CD8+/CD44+/CD62L-); EM, effector memory (CD45+/CD3+/CD8+/CD44+/CD62L-); naive (CD45+/CD3+/CD8+/CD44+/CD62L-); revertant (CD45+/CD3+/CD8+/CD44+/CD62L-).
We next characterized TIL populations [CD3\(^+\), FOXP3\(^+\) (Treg), and CD8\(^+\) T cells] in 4T1 tumor sections by IHC. Total numbers of CD3\(^+\)/FOXP3\(^+\) Tregs were lower in aged mice and unchanged with treatment (Fig. 3G–I). Moreover, CD8\(^+\) T cells in these tumor sections were not elevated with ICB treatment in the aged mice (Fig. 3J and K). These results translated to a significantly increased CD8\(^+\)-Treg ratio in the aged mice (Fig. 3L), but when considered collectively, our results suggested this ratio was more a function of lower Treg numbers rather than a robust CD8\(^+\) T-cell response. In contrast, both CD8\(^+\) T cells and Tregs increased with ICB therapy, particularly with anti-CTLA4 treatment, in young mice (Fig. 3G–L).

We therefore scored intratumoral (T), stromally restricted (S), and peripherally restricted (P) localization of CD8\(^+\) cells and Tregs increased with ICB therapy, particularly with anti-CTLA4 treatment, in young mice (Fig. 3G–L).

“minor infiltration” (S or P > T), “medium infiltration” (T > S), and “hot” (T > S or P). Tumors from both young and aged isotype-treated mice displayed mixed phenotypes by this scoring method and were not significantly different from one another (Fig. 3M). After ICB treatment, tumors from young mice were approximately 3-fold enriched for the “medium” phenotype, whereas “cold” and “minor” phenotypes disappeared (Fig. 3M). In sharp contrast, tumors from aged 4T1 tumor-bearing mice treated with anti–PD-L1 or anti–CTLA4 failed to expand the “medium” phenotype, and in fact emergence of “cold” phenotypes lacking TILs became obvious (Fig. 3M).

Further characterization of TILs revealed that PD-1\(^+\)/TIM3\(^+\) CD8\(^+\) T cells increased approximately 3 to 5-fold with ICB treatment in young mice (Fig. 3N; Supplementary Fig. S3D). Although ICB treatment did not enhance the PD-1\(^+\)/TIM3\(^+\) CD8\(^+\) T-cell population in aged mice, these cells were already

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Figure 4. TNBC tumors of aged mice are deficient for IFN production and response and lack an inflamed phenotype. A–F, GSEA of RNA-seq data showing significantly enriched hallmark signatures when comparing anti–PD-L1 to isotype-treated young mice (n = 10 and 7 mice, respectively; A), anti–CTLA4 to isotype-treated young mice (n = 12 and 7 mice, respectively; B), anti–PD-L1 to isotype-treated aged mice (n = 8 and 7 mice, respectively; C), anti–CTLA4 to isotype-treated aged mice (n = 8 and 7 mice, respectively; D), anti–PD-L1 responders to nonresponders in the young cohort (n = 6 and 4 mice, respectively; E), anti–CTLA4 responders to nonresponders in the young cohort (n = 7 and 5 mice, respectively; F). (continued on following page)
approximately 7-fold elevated in tumors from aged isotype-treated mice relative to young isotype-treated mice (Fig. 3N; Supplementary Fig. S3D). PD-1+ TILs in 4T1 tumors from aged mice also demonstrated a memory-like phenotype particularly after ICB therapy (Fig. 3N; Supplementary Fig. S3D). Overall these data demonstrated that ICB therapy does very little to change the immune contexture of the TME in aged mice.

Age-Dependent Deficiency in IFN Production and Response

To gain further insights into the tumor immune microenvironment at the molecular level that could explain lack of response to ICB therapy in aged mice, we performed whole-tissue RNA sequencing (RNA-seq) on tumors from young and aged mice treated with isotype, anti–PD-L1, or anti-CTLA4 (GSE130472). Multiple genes were significantly differentially expressed between young and aged cohorts in response to treatment, and hierarchical clustering of these genes revealed strong associations based on age and treatment, indicating consistency of effect (Supplementary Fig. S4A and S4B; Supplementary File S1). Gene Set Enrichment Analysis (GSEA) revealed that some of the enriched gene sets shared in common between young and aged mice included UV response and epithelial–mesenchymal transition (EMT) with anti–PD-L1 (Supplementary Fig. S4C and S4D) and TNFα signaling and EMT with anti-CTLA4 (Supplementary Fig. S4E and S4F).

The most significantly enriched signatures in young mice treated with anti–PD-L1 or anti-CTLA4 compared with the isotype control cohorts included gene sets involved in IFNγ response and inflammatory response (Fig. 4A and B). In contrast,
these same signatures were not enriched in the tumors from aged mice with either ICB treatment (Fig. 4C and D). Moreover, when we stratified the young ICB treatment cohorts into responders and nonresponders, IFNγ response, IFNα response, inflammatory response, and allograft rejection were significantly enriched in the responders (Fig. 4E and F; Supplementary Fig. S4G and S4H).

We calculated 24 immune cell–related gene expression signatures via the nCounter Mouse PanCancer Immune Profiling Panel from NanoString and compared relative activity of each signature across all samples (see Methods). Tumors from young mice treated with either anti–PD-L1 or anti-CTLA4 were largely enriched for both innate and adaptive immune cell signatures compared with the young isotype-treated mice (Fig. 4G). However, these immune signatures were not enriched in the aged mice treated with anti–PD-L1 or anti-CTLA4 compared with the respective isotype-treated cohort (Fig. 4G).

Intracellular flow cytometric analysis of CD8+ TILs was consistent with these findings. Specifically, tumors from aged mice had approximately 9-fold reduction in CD8+/IFNγ cells than those from young mice, and numbers of these cells were not increased with ICB treatment as they were in young mice (Fig. 4H). Moreover, CD8+ T cells in tumors from aged mice stored significantly more of the cytotoxic granule component granzyme B (GrzB) in response to either ICB treatment, which was not evident in the young ICB-treated mice (Fig. 4I).

Given that IFNγ production and release of GrzB are critical for antitumor immune responses, our data indicated that ICB was less effective at triggering antitumor responses in aged mice. Moreover, our results established an important correlation between expression of inflammatory response and IFN signaling pathways in the TME and the ability of TNBC tumors to respond to ICB therapy.

Baseline IFN Production and Response Genes Stratify with Age in Mice and Patients with TNBC

We next asked whether age-dependent differences in critical ICB response pathways would be evident even prior to treatment. Principal component analysis (PCA) of the RNA-seq data from murine tumors confirmed that tumors from young and aged isotype-treated mice formed distinct clusters that stratified with age (Fig. 5A). Strikingly, GSEA revealed that the tumors from young isotype-treated mice were enriched for IFNα, IFNγ, and inflammatory response genes relative to those from aged isotype-treated mice (Fig. 5B). We also applied a previously reported IFNγ pathway signature that defined response to anti-CTLA4 in patients with melanoma (defined as Gao_Signature; ref. 26). Again, we found that tumors from young isotype-treated mice were enriched for this IFN response pathway compared with tumors from aged mice (Fig. 5B).

Processing and presentation of tumor antigens is also critical for initiating antitumor immune responses. Therefore, we also examined genes associated with antigen processing and presentation and found significant differences in expression of a number of these genes between young and aged isotype-treated mice (Fig. 5C).

Together, these results established that the critical mediators of response to ICB therapy could be prospectively identified in tumor gene expression data even prior to treatment. We therefore interrogated the METABRIC database for patients with TNBC and stratified them into two groups based on age at initial diagnosis: ≤40 and ≥65 years of age. Two distinct clusters of the most differentially expressed genes were observed in patients ≤40, whereas these genes did not cluster in the patients ≥65 (Supplementary Fig. S5).

In patients ≤40, IFNα response, IFNγ response, inflammatory response, allograft rejection, IL6–JAK–STAT3 signaling, and IL2–STAT5 signaling gene sets were the most highly enriched relative to those ≥65 (Fig. 5D), similar to our preclinical observations. To further understand which immune cell subtypes may be associated with these changes, we applied CIBERSORT to characterize 22 inferred immune subsets for each patient (27). In patients ≥65 relative to those ≤40, M1 macrophage and plasma cell signatures were significantly reduced whereas activated natural killer (NK) cells and M0 macrophage signatures were significantly enriched (Fig. 5E).

Taken together, these results established that the immune contexture of the TME in TNBC is significantly different between young and older patients, suggesting that age-related immune changes may lead to reduced efficacy of ICB in older individuals.

Stimulating IFN Signaling Improves Outcome and Response to ICB in Aged Mice

Our results raised the question of whether stimulation of IFN signaling, which was enriched in young mice but lacking in aged mice, is sufficient to trigger response to ICB in aged mice. In particular, type I interferons (IFNα and IFNβ) play an important role in T-cell priming, and our results suggested that failed innate immune priming results in the lack of response to ICB in aged mice. Activation of the stimulator of interferon genes (STING) pathway promotes the transcription of type I IFNs and has previously been reported to promote antitumor immunity in other preclinical models (28).

Figure 5. Age-dependent deficiencies in IFN production and response, inflammatory responses, and antigen presentation in mice and patients with TNBC. A, PCA of RNA-seq data from 411 tumors of young and aged isotype-treated mice (n = 7 tumors/cohort; 1 tumor per mouse) B, GSEA of significantly enriched hallmark signatures in young relative to aged isotype-treated mice (n = 7 tumors/cohort; 1 tumor per mouse) C, The log2 fold change and adjusted P value of differentially expressed genes related to antigen processing and presentation in tumors from young and aged isotype-treated mice D, GSEA of the significantly enriched hallmark pathways in tumor tissues from patients with TNBC ≥65 years (n = 49) relative to those ≤40 years (n = 68) from the METABRIC database. E, Quantification of the CIBERSORT immune cell signatures applied to the METABRIC database comparing tumors from patients with TNBC ≥60 years (n = 49) relative to those ≤40 years (n = 68). Two-tailed unpaired t test with Welch correction (E). Error bars, SEM. *, P < 0.05.
Age and Response to Immune Checkpoint Blockade in TNBC

A

B

Young isotype vs. aged isotype

Enrichment plot:

HALLMARK_INTERFERON_ALPHA_RESPONSE

NOM P < 0.000

FDR q < 0.000

NES = 1.66

ES = 0.58

NES = 2.40

Patients with triple-negative breast cancer ≤40 vs. ≥65

Enrichment plot:

HALLMARK_INTERFERON_GAMMA_RESPONSE

NOM P < 0.000

FDR q < 0.000

NES = 1.48

ES = 0.36

NES = 1.52

Enrichment plot:

HALLMARK_INFLAMMATORY_RESPONSE

NOM P = 0.05

FDR q = 0.155

ES = 0.27

NES = 1.25

Enrichment plot:

HALLMARK_INTERFERON_GAMMA_RESPONSE

NOM P < 0.000

FDR q < 0.000

NES = 1.81

ES = 0.44

NES = 2.08

Enrichment plot:

HALLMARK_ALLOGRAFT_REJECTION

NOM P = 0.007

FDR q = 0.000

ES = 0.507

NES = 2.08
To enhance IFN signaling, we used intratumoral injection of the mouse-specific STING agonist 5,6-dimethylxanthenone-4-acetic acid (DMXAA).

We first tested DMXAA monotherapy and combination with either anti–PD-L1 or anti–CTLA4 in aged BALB/c mice with 4T1 TNBC, according to our protocol (Supplementary Fig. S6A–S6D). As observed repeatedly, neither anti–CTLA4 nor anti–PD-L1 monotherapy was efficacious in aged mice (Fig. 6A–F; Supplementary Fig. S6E–S6J). DMXAA monotherapy significantly slowed tumor growth only during the course of treatment (Fig. 6A, B, D, and E; Supplementary Fig. S6E–S6H) but did not significantly improve overall survival (Fig. 6C and F). Importantly, both DMXAA + anti–PD-L1 and DMXAA + anti–CTLA4 combination therapies significantly reduced 4T1 tumor growth, resulting in significantly improved overall survival of aged mice (Fig. 6A–F; Supplementary Fig. S6E–S6H).

Interestingly, in four independent experiments in young mice, DMXAA monotherapy did not alter tumor growth relative to the isotype control cohort (Fig. 6G; Supplementary Fig. S6A–S6D). As observed repeatedly, neither anti-CTLA4 with 4T1 TNBC, according to our protocol (Supplementary Fig. S6E–S6H). DMXAA monotherapy was performed in three independent experiments; combination treatment was performed twice. B, Fold change in tumor volume during the course of indicated treatment (n = 4–7 mice per group/COHORT). C, Kaplan–Meier survival analysis of aged 4T1 tumor-bearing mice treated with isotype, anti–PD-L1, DMXAA, or combination DMXAA + anti–PD-L1 (n = 5–8 mice/COHORT). D, Growth kinetics of 4T1 tumors in aged mice treated with isotype (100 μg), anti–PD-L1 (100 μg), DMXAA (250 μg), or the combination DMXAA (250 μg) + anti–PD-L1 (100 μg; n = 5–8 mice/COHORT). E, Fold change in tumor volume during the course of indicated treatment (n = 4–11 mice/COHORT). F, Kaplan–Meier survival analysis of aged 4T1 tumor-bearing mice treated with isotype, anti–PD-L1, DMXAA, or combination DMXAA + anti–PD-L1 (n = 5–11 mice/COHORT). Growth kinetics (G) of 4T1 tumors from young mice treated with isotype (100 μg), anti–CTLA4 (100 μg), DMXAA (250 μg), or DMXAA (250 μg) + anti–CTLA4 (100 μg). The combination therapy group received 3 doses of anti–CTLA4 in this experiment (n = 5–7 mice per group, representing one experiment). H and I, qPCR analysis of the expression of Ifna1 (H) and Ifnb1 (I) relative to GAPDH from vehicle (DMSO) or DMXAA (250 μg) treated 4T1 tumor-bearing young and aged mice (n = 6 mice per group, representing one experiment). One-way ANOVA corrected for multiple corrections (H and I) and log-rank (Mantel–Cox) test (C and F). Error bars, SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

**Figure 6.** Stimulating IFN signaling improves response to ICB and overall survival in aged mice. A, Growth kinetics of 4T1 tumors in aged BALB/c mice treated with isotype (100 μg), anti–CTLA4 (100 μg), DMXAA (250 μg), or the combination DMXAA (250 μg) + anti–CTLA4 (100 μg; n = 5–8 mice/COHORT). DMXAA monotherapy was performed in three independent experiments; combination treatment was performed twice. B, Fold change in tumor volume during the course of indicated treatment (n = 4–8 mice/COHORT). D, Growth kinetics of 4T1 tumors in aged mice treated with isotype (100 μg), anti–PD-L1 (100 μg), DMXAA (250 μg), or the combination DMXAA (250 μg) + anti–PD-L1 (100 μg; n = 5–8 mice/COHORT). E, Fold change in tumor volume during the course of indicated treatment (n = 4–11 mice/COHORT). F, Kaplan–Meier survival analysis of aged 4T1 tumor-bearing mice treated with isotype, anti–PD-L1, DMXAA, or combination DMXAA + anti–PD-L1 (n = 5–11 mice/COHORT). Growth kinetics (G) of 4T1 tumors from young mice treated with isotype (100 μg), anti–CTLA4 (100 μg), DMXAA (250 μg), or DMXAA (250 μg) + anti–CTLA4 (100 μg). The combination therapy group received 3 doses of anti–CTLA4 in this experiment (n = 5–7 mice per group, representing one experiment). H and I, qPCR analysis of the expression of Ifna1 (H) and Ifnb1 (I) relative to GAPDH from vehicle (DMSO) or DMXAA (250 μg) treated 4T1 tumor-bearing young and aged mice (n = 6 mice per group, representing one experiment). One-way ANOVA corrected for multiple corrections (A, B, D, E, and G). Two-sided unpaired t test with Welch correction (H and I) and log-rank (Mantel–Cox) test (C and F). Error bars, SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Fig. S6I and S6K). Anti-CTLA4 and the combination DMXAA + anti-CTLA4 significantly reduced tumor growth to the same degree in the young mice, suggesting there is no additive effect of DMXAA in young mice (Fig. 6G; Supplementary Fig. S6J and S6L).

To ensure that DMXAA treatment was affecting type I IFN expression, we performed qPCR on whole tumors one day following intratumoral injection of DMXAA (Supplementary Fig. S6M and S6N). Importantly, expression of both IFNα and IFNβ was significantly enhanced after DMXAA injection in both young and aged mice, with no age-associated differences in relative expression observed (Fig. 6H and I). Together, these results established that response to ICB in aged mice with TNBC is rescued by stimulating type I IFNs within the tumor microenvironment.

**DISCUSSION**

This work revealed a critical role for physiologic aging in reducing ICB efficacy in murine models of TNBC. Tumors from young mice with TNBC and patients with TNBC <40 years display enrichment of genes associated with antigen presentation, inflammation, and IFN response pathways, suggesting efficient innate priming of CD8+ T cells (Fig. 7, top left). In young mice, these pathways are further enriched with ICB therapy and together with increased infiltration of CD8+ T cells this results in a robust antitumor response (Fig. 7, top right). Tumors from aged mice and patients with TNBC >65 years lack enrichment for genes associated with antigen presentation, inflammation, or IFN response pathways, suggesting that T-cell priming by antigen-presenting cells is not as efficient as in young mice (Fig. 7, bottom left). Aging also results in an immunologically cold tumor microenvironment, defined by peripherally restricted TILs that have increased checkpoint protein expression (Fig. 7, middle). The age-dependent tumor microenvironment fails to generate an antitumor response to ICB (Fig. 7, bottom right). Stimulating type I IFN signaling with a STING agonist (DMXAA) promotes response to ICB in the aged mice (Fig. 7, bottom right).

To date, only a handful of studies have investigated age-related immune dysfunction as a potential mechanism for resistance to ICB. In advanced NSCLC, where anti–PD-1 antibodies are now standard-of-care for patients who progress on or after platinum-based chemotherapy, patients >75 years of age demonstrated less benefit after nivolumab therapy compared...
with younger patients; however, overall survival (OS) and toxicity were not different (29–32). One meta-analysis of data from more than 5,000 patients treated with ICB found no difference in OS between young and older patients in multiple cancer types (33). Another meta-analysis with greater representation of patients more than 65 years of age also showed comparable efficacy of anti–PD-1 or anti–PD-L1 therapy in young and older patients (34). In addition, comparative benefit and toxicity analyses have shown only modestly higher rates of treatment discontinuation and immune-related adverse events in patients with melanoma older than 80 years of age when treated with ICB (35). Conversely, another recent study showed increased rates of response to ICB in older patients with melanoma (36).

It is worth noting that most of the studies of age and response to ICB, be it patient data or mouse models, have been done in NSCLC or melanoma. These cancer types generally have a higher mutational load and increased immunogenicity compared with breast cancer (37), which, among other things, makes them more susceptible to ICB, which is now part of standard-of-care therapy in these cancers. In fact, it is now well accepted that tissue-specific differences in the immune microenvironment critically affect cancer progression and therapeutic responses (38). Likewise, mouse strain and life span, tumor models, complexity of the immune microenvironment, and specific ICB therapy are important considerations when drawing conclusions from preclinical studies. For example, decreased numbers of tumor-infiltrating Tregs were associated with improved response to anti–PD-1 therapy in 10-month-old C57BL/6 mice in a recent melanoma study (36). However, decreased Tregs in the 4T1 tumors from aged (>12 months) compared with young BALB/c mice did not lead to improved responses to anti–PD-L1 or anti–CTLA4 in our study. In fact, decreased Tregs with age appeared to be a function of a “cold” immune environment in our models, which is supported by our observation of reduced IFN signatures with age. Importantly, the young mice bearing either 4T1 or Met1 tumors responded to ICB, unlike young mice in the melanoma study (36). Conversely, the 4T1 tumor model results in neutrophilia in BALB/c mice, which can also limit the effects of ICB in general, although we observed that intratumoral neutrophils did not change in young compared with aged mice, whereas response to ICB did. Therefore, although neutrophilia may contribute to the overall limited immunogenicity of the 4T1 model, it does not explain the age-specific response to ICB we observed, or that intratumoral stimulation of type I IFNs could recover responses to ICB in aged mice. Clearly, however, the caveats to preclinical models highlight the need for more detailed clinical data from ICB trials in different cancers.

The application of ICB to TNBC is still under active investigation, with atezolizumab in combination with abraxane recently receiving FDA approval for first-line treatment of metastatic TNBC (13). ICB monotherapy in breast cancer shows response rates of only 5% to 10% in unselected cohorts (12, 39, 40). In selected cohorts of patients with PD-L1+ metastatic TNBC in the phase II KEYNOTE 086 trial, pembrolizumab as a first-line therapy showed an objective response rate (ORR) of 21.4% (41). Although this result was not significant, the authors note the ORR was numerically higher in patients who were >65 or postmenopausal. This difference was not as apparent when a cutoff of >50 was used. Age stratification in the Impassion130 trial showed that both young (18–40 years) and older (>65 years) women performed better with combination therapy (18–40: 3.7 vs. 3.6 months; >65: 9.1 vs. 6.2 months; ref. 13). However, as with nearly all breast cancer clinical trials, young and older women were underrepresented (12.6% and 24%, respectively) in this trial. TNBC also presents unique challenges to ICB therapy due to age-associated differences in disease progression as well as the composition of the tumor microenvironment (42), as we, and others, have reported (43).

Correlative studies from TNBC trials of ICB that include longitudinal molecular and histopathologic assessment have not yet been reported. Therefore, the baseline immune phenotype of tumors from the reported studies is unknown, making interpretation of their results difficult in the context of our data. Interpretation of these types of analyses are further complicated by the fact that we do not actually know where the appropriate cutoff for “old” is; therefore, grouping all patients above or below a certain age may mask any actual age-specific effects. In fact, a recent study demonstrated considerable differences in the rate of immune aging between healthy individuals (24), supporting the notion that immune age does not necessarily reflect chronological age, and that in fact immunologic age may be a better predictor of response to immunotherapy.

The age-stratified responses to ICB that we observed in our TNBC mouse models appear to be due to defective innate immune priming with age, as tumors from aged mice (without therapy) showed overall decreased antigen processing and presentation as well as decreased IFNγ and IFNγ signaling, resulting in an overall “cold” TME that was not responsive to ICB monotherapy. Only the addition of the STING agonist DMXAA, which drives type I IFN signaling, showed efficacy in combination with ICB in aged mice. Our parallel findings in tumors from older patients with TNBC in the METABRIC cohort suggest that older patients with TNBC exhibit a “cold” TME characterized by poor T-cell infiltration and a lack of IFN signaling. To the best of our knowledge, these data demonstrate for the first time that older age is a defining factor in determining the T cell–inflamed phenotype in TNBC and could be an important determinant of ICB therapy response.

Intratumoral expression of IFN pathway genes is closely associated with response to ICB in the clinic. Intratumoral loss of IFNγ signaling is associated with primary resistance to anti–CTLA4 therapy (26), whereas a T cell–inflamed TME characterized by active IFNγ signaling and antigen presentation is a common feature associated with tumors that are responsive to anti–PD-1 (44). In line with these studies, our data suggest that analysis of IFN-related genes taken from tumor genomic data may provide prognostic utility for patients selected to receive ICB monotherapy or potentially ICB therapy in combination with an IFN pathway activator, such as a STING agonist, to generate a T-cell response in “cold” tumors.

Clinical trials utilizing intratumoral injection of synthetic CDN STING agonists alone or in combination with anti–PD-1...
are now under way. One of these trials (NCT03010176) using the STING agonist MK-1454 also includes patients with TNBC, and early reports suggest the combination with pembrolizumab is more effective than MK-1454 alone, which lacked efficacy. Likewise, our study indicated that the mouse STING agonist DMXAA as monotherapy did not affect tumor growth in young mice, but significantly improved response to ICB in aged mice. Given these results, one might speculate that efficacy of STING agonists stratifies with age or that patients whose tumors lack enrichment for IFN signatures would benefit from these therapies. Nevertheless, the implications of our findings for patients with TNBC whose tumors reflect the biology we observed have yet to be determined.

A critical aspect of effective T-cell responses is antigen presentation, primarily via professional antigen-presenting cells, including macrophages and DCs. Macrophages have been shown to exhibit decreased antigen presentation and MHC Class II expression with age (45). Notably, we observed decreased expression of antigen processing and presentation genes in tumors from aged mice. In line with these observations, we provide gene expression–based evidence of reduced tumor-infiltrating M1 macrophages in older patients with TNBC in our analysis of the METABRIC cohort, and in TNBC tumors from aged mice compared with young. The ability of DCs to prime T cells (among other functions) has also been reported to decrease with age (46). This aspect of tumor immunology remains to be further investigated in our models.

Although ICB therapy presents an emerging treatment option for older patients with TNBC and older patients with cancer in general in terms of efficacy and safety compared with chemotherapy, how the aging immune system responds in cancer and, more specifically, after ICB treatment, remains poorly understood. Immune-related adverse events and ICB-related toxicities may also worsen with age, but the lack of elderly patients enrolled in clinical trials hinders progress in this area. With an aging global population predicted to rise from 10% in 2000 to more than 22% by 2050 (47), this is an increasingly pressing concern. Building on the preclinical results and data presented here, it may be possible to stratify patients with TNBC into those requiring combination therapy with an immune-stimulatory agent such as a STING agonist using tumor genomic data for IFN pathway genes, and therefore make ICB therapy applicable to patients with TNBC of all ages.

METHODS

Animals

Female BALB/c ByJ, FVB/NJ, C57BL/6, and NCR-NU (nude) mice were purchased from Jackson Labs. BALB/c mice were aged in house or provided by the National Institute on Aging at 12 months of age. FVB/NJ and NCR-NU mice were aged in house. For all experiments, young mice were defined as 8 to 12 weeks of age; aged mice as >12 months of age. All experiments were conducted in accordance with regulations of the Children’s Hospital Institutional Animal Care and Use Committee (protocol # 12-11-2308RR) and the Brigham and Women’s Hospital (protocol # 2017N000056).

Cell Lines

4T1 tumor cells were provided by F. Miller (Wayne State University School of Medicine) and maintained as described previously (48). The Met1 TNBC cell line was a gift from J. Joyce (University of Lausanne) with permission from A. Borowsky (UC Davis School of Medicine) and maintained as described previously (49). The McNeuA cell line was provided by Michael Campbell (University of California, San Francisco) and maintained as described previously (50). The 4T07 and 67NR cell lines were provided by Robert Weinberg (Whitehead Institute for Biomedical Research and MIT) and maintained as described previously (51). Met1 cells were generated from a spontaneously arising tumor in an FVB/N-Tg (MMTV-PyVmT) mouse (49) and 4T1 cells are a variant line from a single spontaneously arising mammary tumor from a BALB/cF3H mouse (52). All cells were routinely tested for Mycoplasma and confirmed negative when used in experiments.

For IFNγ stimulation to assess PD-L1 and MHC I expression, tumor cells were incubated for 24 hours with 1 ng/mL recombinant mouse IFNγ (BioLegend, 575304) and expression of cell surface receptors was analyzed by flow cytometry.

Tumor Models

Tumor cells were prepared in sterile PBS and injected orthotopically into the fourth left mammary fat pad (1 × 10^5 4T1, 1 × 10^4 4T07, and 1 × 10^6 67NR cells in BALB/c or nude mice; 2 × 10^5 Met1 or 1 × 10^6 McNeuA in FVB or nude mice) on day 0. Mice were measured every second or third day using digital calipers, and tumor volume was calculated as π x length x width^2 / 6. For single-agent immune checkpoint inhibition, 100 or 200 μg anti-CTLA4 (clone 9D9, BioXCell, BE0164), anti–PD-L1 (clone 10F.9G2, BioXCell, BE0101), or isotype (clone LTF-2, BioXCell, BE0090) antibodies in 100 or 200 μL sterile PBS were used. All ICB mAbs were injected intraperitoneally at three-day intervals for a total of four doses, starting at day 7 (4T1 and Met1) post-tumor cell injection when tumors reached a volume of no more than approximately 5 mm^3 in all mice. For survival studies, mice were considered at an ethical endpoint when the tumors reached 800 mm^3 in volume.

For CD8 T-cell depletion studies, 100 μg anti-CD8β (clone 53-5.8, BioXCell, BE0223) was prepared in 100 μL sterile PBS and was administered intraperitoneally on days 6, 7, then weekly until experimental endpoint. Single-agent ICB was administered as described above. CD8 T-cell depletion from the blood was confirmed by flow cytometry on day 8 post tumor cell injection.

For combination DMXAA and immune checkpoint inhibition experiments, 250 μg DMXAA (Invivogen, tlrl-dmx) was injected intracranially on day 10 and 100 μg of either anti–PD-L1 or anti–CTLA4 was prepared in 100 μL sterile PBS and administered intraperitoneally every three days for a total of 4 doses starting on day 10 post tumor cell injection. For single-agent ICB, 100 μg of ICB mAb was prepared in 100 μL of sterile PBS and injected intraperitoneally starting at day 7 post tumor cell injection unless otherwise stated.

For RNA-seq experiments, tumor cells were injected bilaterally into the fourth left and right mammary fat pads then treated with 3 doses of ICB. Tumors were excised one day after the third dose of treatment (day 14) to ensure the capture of an antitumor response. The mice were considered nonresponders when tumor growth increased regardless of age, partial responders when tumor growth remained similar with treatment, and responders when tumor growth reduced with treatment.

Hemovet

Blood was collected by either retro-orbital or a terminal intracardiac bleed into EDTA-coated tubes. Blood samples were analyzed on a Hemavet 950 (Drew Scientific) according to the manufacturer’s instructions.

Flow Cytometry

Tumors were excised on day 19 of tumor growth after receiving 4 doses of ICB, cut into small pieces, and digested with 0.2 mg/mL
collagenase type IV (Worthington Biochemical Corp. LS004186) and 0.02 mg/mL DNase I (Roche, 04536282001) for 1 hour at 37°C with agitation. Single-cell suspensions were generated from tumors, blood, and spleens and incubated with anti-CD16/32 for 20 minutes on ice to block Fc receptors, and relevant antibodies for 30 minutes on ice. For intracellular staining, samples were fixed and permeabilized using the Focpx3 fixation/permeabilization kit (eBioscience, 00–5523–00) according to the manufacturer’s instructions. Nonviable cells were excluded on the basis of staining with 7-aminoactinomycin D (BioLegend, 420404), or Zombie Yellow for intracellular staining (BioLegend, 423104). CountBright absolute counting beads (Life Technologies, C36950) were used to calculate absolute cell numbers according to the manufacturer’s instructions. Flow cytometry acquisition was carried out on a FACs Fortessa or LSR II (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star). Antibody details are listed in Supplementary Table S1.

**T-cell Stimulation**

CD8+ T cells were isolated from the spleens and lymph nodes of young and aged mice using autoMACS and the CD8 T Cell Negative Selection Kit (Miltenyi Biotec, 130-104-075) according to the manufacturer’s instructions. T cells were labeled with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (Sigma, 21888) for 10 minutes at room temperature and 1 × 10^6 cells incubated at a 2:1 ratio with CD3/CD28 magnetic beads (Dynabeads, Gibco, 11452D) for 72 hours before analysis by flow cytometry.

**IHC and Microscopy**

Dissected tissues were fixed in 4% paraformaldehyde for 24 hours, stored in 70% ethanol for 24 hours, paraffin-embedded, and sectioned. Sectioning and hematoxylin and eosin, F4/80, Ki67, and MPO staining were performed by the DF/HCC Specialized Histopathology Core at Bgham and Women’s Hospital. IHC was performed as described previously (53), using citrate buffer for antigen retrieval and staining with primary antibodies at 4°C overnight. Images were captured using a Nikon Eclipse Ni microscope, and a minimum of four images per tumor were analyzed by two independent investigators in a blinded fashion. CD3+ T cells, CD8+ T cells, and FOXP3+ Tregs were counted using ImageJ; eSMA, F4/80, and MPO stains using CellProfiler as described previously (54); and Ki67 staining using the ImmunoRatio program (55). Antibody details are listed in Supplementary Table S1.

The localization of CD8+ T cells was scored blindly by two independent investigators on a 0–5 scale where 0 is cold (no TILs), 1 is restricted (peripheral or stromal only), 2 is minor infiltration (S or S), 3 is medium infiltration (T=S), and 5 is hot (significant TILs).

**RNA Isolation and Sequencing**

RNA was extracted from tumors no larger than approximately 150 mm^3 and stored in 1 mL RNALater (Qiagen, 1018087) at 4°C overnight before being transferred to −80°C. Tumors were homogenized using a homogenizer 150 (Thermo Fisher Scientific) in RLT buffer (Qiagen, 79216), processed via QIAshredder columns (Qiagen, 79656), and then total RNA was isolated using an RNeasy Mini Kit (Qiagen, 74136) according to the manufacturer’s protocol. Quality of total RNA was determined using a 2100 Bioanalyzer (Agilent). RNA-seq was performed using paired-end sequencing with a read length of 75bp on the Illumina HiSeq 2000 platform by the Dana-Farber Cancer Institute Molecular Biology Facility. All data are deposited in the Gene Expression Omnibus (GSE130472).

**RNA-seq Differential Expression and Functional Enrichment Analysis**

All samples were processed and analyzed using an RNA-seq pipeline implemented in the bebo-nextgen project (https://bebo-nextgen.readthedocs.org/en/latest/) and custom R scripts. Reads were aligned to the Genome Reference Consortium Mouse Reference build number 38 (GRCm38) of the mouse genome (aka mm10) augmented with transcript information from Ensembl using STAR (56) with soft trimming enabled to remove adapter sequences, other contaminant sequences such as poly-A tails and low Phred quality score sequences. Counts of reads aligning to known genes were generated by featureCounts (57) for use in quality-control measures. In parallel, transcripts per million measurements per isoform were generated by quasi-alignment using Salmon (58) for use in clustering and differential expression analyses. STAR alignments were checked for evenness of coverage, ribosomal RNA content, genomic context of alignments (for example, alignments to exons and introns), complexity, and other quality checks using a combination of FastQC, Qualimap (59), MultiQC (https://github.com/ewels/MultiQC), and custom tools. Samples were clustered in an unsupervised manner by both PCA and hierarchical means using rlog-transformed reads to identify potential outliers and technical artefacts. All samples passed these quality-control measures. Differential expression at the gene level was called with DESeq2 (60), using the counts per gene estimated from the Salmon quasi-alignments by tswift (61) as quantitating at the isoform level has been shown to produce more accurate results at the gene level.

Heat maps of expression levels were generated with heatmap2 (62), centered (mean of row subtracted from all values) and scaled (all centered values divided by the SD of the row), and then clustered via the ward.D2 algorithm (63).

Cutoff-free GSEA was also performed against Gene Ontology terms, restricting the analysis to those terms with at least 10 genes, using clusterProfiler (64) and the ranked log, fold changes of all genes from the DESeq2 analyses. Functional gene sets were considered enriched if their FDR-adjusted P value was less than 0.05.

The nCounter Mouse PanCancer Immune Profiling Panel from NanoString was used for the relative expression of immune cell signatures. For each cell type, the expression of all the associated genes was averaged and used as cell type enrichment measure. These values were used for the heat map plots.

GSEA was performed using the Hallmark gene set using version 3 of the GSEA software (Broad). The number of permutations was set to 1,000 and the permutation type was gene-set. Results were considered significant with a P value and FDR value under 0.05.

**qPCR**

RNA was isolated from whole tumors as described above, and the concentration was determined using a Nanodrop (Thermo Fisher Scientific). cDNA was synthesized using the SuperScript Reverse Transcription Supermix for qRT-PCR according to the manufacturer’s specifications (Bio-Rad, 170-8841) and generated on a T100 Thermal Cycler (Bio-Rad). qPCR amplification was performed using iTaq Universal SYBR Green supermix (Bio-Rad, 1725124) and run on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Relative gene expression was quantitated using the ∆∆C value with the following primers: IFNα1: (forward-5′-GCTAGCGGTCTGTTCTTCT-3′; reverse-5′-TCTCGCCGGAATCAAGTC-3′); IFNβ1: (forward-5′-ACGACAGGCCCTTTCTCAATC-3′; reverse-5′-TTGAAAGTCCGCCCCTG-3′); STING: (forward-5′-CCAAGACCCACACAGGGAA-3′; reverse-5′-GCTCTTGGGACAGTACGGAG-3′); GAPDH (forward-5′-GGTGAGGTCGTTGAACGC-3′; reverse-5′-CTCCGCCTCTGGAGTGGTG-3′). All reactions were performed in duplicate and expression levels were normalized to the housekeeping gene GAPDH.

**Human Data Analysis**

Normalized gene expression data and paired clinical feature data were obtained from the METABRIC European Genome-phenome
Age and Response to Immune Checkpoint Blockade in TNBC

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REFERENCES

Age and Response to Immune Checkpoint Blockade in TNBC

Archive (IDs EGAD00010000210 and EGAD00010000221). TNBC was defined as tumors negative for estrogen receptor, progesterone receptor, and HER2 amplification by SNP6 analyses. Patients were stratified into “40 and younger” (age at diagnosis ≤40) versus “65 and over” (age at diagnosis ≥65). GSEA was performed using the Hallmark gene set using version 3 of the GSEA software (Broad). The number of permutations was set to 1,000 and the permutation type was gene-set. Results were considered significant with a P value and FDR value under 0.05. Inferred immune subset proportion was determined via CIBERSORT (27), using the LM22 dataset.

Statistical Analysis
The data are represented as mean ± SEM. The data was analyzed by two-way ANOVA with the Sidak multiple comparison correction, one-way ANOVA with Tukey multiple comparison test for significance, or an unpaired two-tailed t test with Welch correction as indicated in the figure legends. Kaplan–Meier survival curves were analyzed using log-rank (Mantel–Cox) test. All the data were analyzed using GraphPad Prism 7.0. P < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest
E.A. Mittendorf is a consultant/advisory board member for Merck, Genomic Health, Sellas Lifesciences, AstraZeneca, and Roche/Gentech. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Sceneay, G.J. Goreczny, K. Wilson, T. Laszewski, D.G. Stower, V. Barrera, J.N. Hutchinson, R.A. Freedman, E.A. Mittendorf, S.S. McAllister
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RESEARCH ARTICLE


Age and Response to Immune Checkpoint Blockade in TNBC

Interferon Signaling Is Diminished with Age and Is Associated with Immune Checkpoint Blockade Efficacy in Triple-Negative Breast Cancer

Jaclyn Sceneay, Gregory J. Goreczny, Kristin Wilson, et al.


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