AWARD NUMBER: W81XWH-20-1-0472

TITLE: Understanding the Impact of Age on Response to Immunotherapy in Breast Cancer

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REPORT DATE: JULY 2021

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
JULY 2021	Annual Progress Report	06/15/2020 – 06/14/2021

4. TITLE AND SUBTITLE

Understanding The Impact of Age on Response to Immunotherapy in Breast Cancer

5a. CONTRACT NUMBER
W81XWH-20-1-0472

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER
0011484619

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Immune checkpoint blockade (ICB) therapy, which is designed to unleash a patient's immune system to attack cancer, has been revolutionary for treating cancer patients; nevertheless, ICB therapy is ineffective for many breast cancer patients. Most breast cancer clinical trials are focused on testing various chemotherapies to identify ones that make ICB work better. However, we believe that there are better ways to improve ICB than to use harsh chemotherapies and that we could identify more effective drugs if we understood more about a breast cancer patient's immune status. We contend that age is an important consideration with respect to ICB, given that normal aging is associated with profound changes to the immune system. Our preliminary studies support the idea that ICB may not be as successful for older breast cancer patients as it is for younger patients and that other treatments need to be studied in order to help older patients. For our project, we proposed to identify existing drugs that improve responses to ICB in aged mice that could eventually be used to help patients. Assessing the immune changes that occur with age could provide a foundation for effectively applying ICB to breast cancer patients of all ages. Ultimately, we hope to provide a basis for improved clinical trial design and to help oncologists make decisions about age-stratified treatment options using ICB for breast cancer. Success in our endeavors has the potential to save lives by delivering the promise of immunotherapy to even more breast cancer patients than are currently seeing benefit.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. **INTRODUCTION:**

So many breast cancer clinical trials are now focused on finding chemotherapy backbones that might improve efficacy of immune checkpoint blockade (ICB), without much rationale. Information about immunological age has not yet entered the conversation, which is surprising given that normal aging is associated with profound changes to the immune system and the median age of breast cancer patients is 62, an age at which immune decline is evident. Our emerging paradigm of age-stratified responses to ICB provides entirely new avenues from which to investigate and improve ICB response. Our objective is to perform pre-clinical work that supports rational trial design replacing harsh chemotherapies with less toxic therapies that improve ICB efficacy and significantly impact survival. We hypothesize that in breast cancer, age presents unique obstacles to ICB efficacy and that understanding age-related changes in immune function will provide a platform for predicting therapeutic response and aid design of improved age-stratified treatment strategies. To test our hypothesis, we proposed the following aims: 1) Identify age-stratified biomarkers of ICB response in TNBC and HR+ breast cancer; 2) Define tumor immunological age in TNBC and HR+ breast cancer as a way to predict ICB response; 3) Evaluate efficacy of pre-clinical ICB combination therapies in young and aged cohorts. We designed experiments using breast cancer cells, pre-clinical age-dependent breast cancer models, and breast cancer patient data and tissue specimens in order to test new therapeutic strategies before evaluating them in patients.

2. **KEYWORDS:**

breast cancer, age, immunologic age, immunotherapy, immune checkpoint blockade, immunosenescence

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

Our project was designed with the following 3 major goals during the entire funding period:

Major Task 1: Identify Age-Stratified Biomarkers of ICB Response in TNBC and HR+ Breast Cancer
(proposed for months 1-30; **30% complete**)

Major Task 2: Define Pre-Treatment Tumor Immunological Age in TNBC and HR+ Breast Cancer as a way to Predict ICB Response
(proposed for months 12-30; **10% complete**)

Major Task 3: Evaluate Efficacy of Pre-clinical ICB Combination Therapies in Young and Aged Cohorts.
(proposed for months 1-20; **50% complete**)

- **What was accomplished under these goals?**

Major Task 1: Identify Age-Stratified Biomarkers of ICB Response in TNBC and HR+ Breast Cancer (months 1-30; 30% complete)

First, we assessed tumor growth in young (10-12 week old) and aged (>12 months old) mice in response to anti-PD-L1 treatment using our pre-clinical models of TNBC and HR+ breast cancer. To do so, we injected 2.5 x10^5 Met1 (TNBC) or 2x10^5 E0771 (HR+ breast cancer) cells orthotopically into young (10-12 week-old) or aged (>12 month-old) FVB or C57BL/6 mice, respectively. Cohorts of mice were randomized into treatment groups (n=5 per group) when tumors reached average 2 mm in size and then mice were administered 4 doses of either isotype (100µg, i.p.), anti-PD-L1 (100µg, i.p.), or anti-CTLA4 (200µg, i.p.). Primary tumor growth was measured using digital calipers beginning on day 7 and every 3 days throughout the experiment.

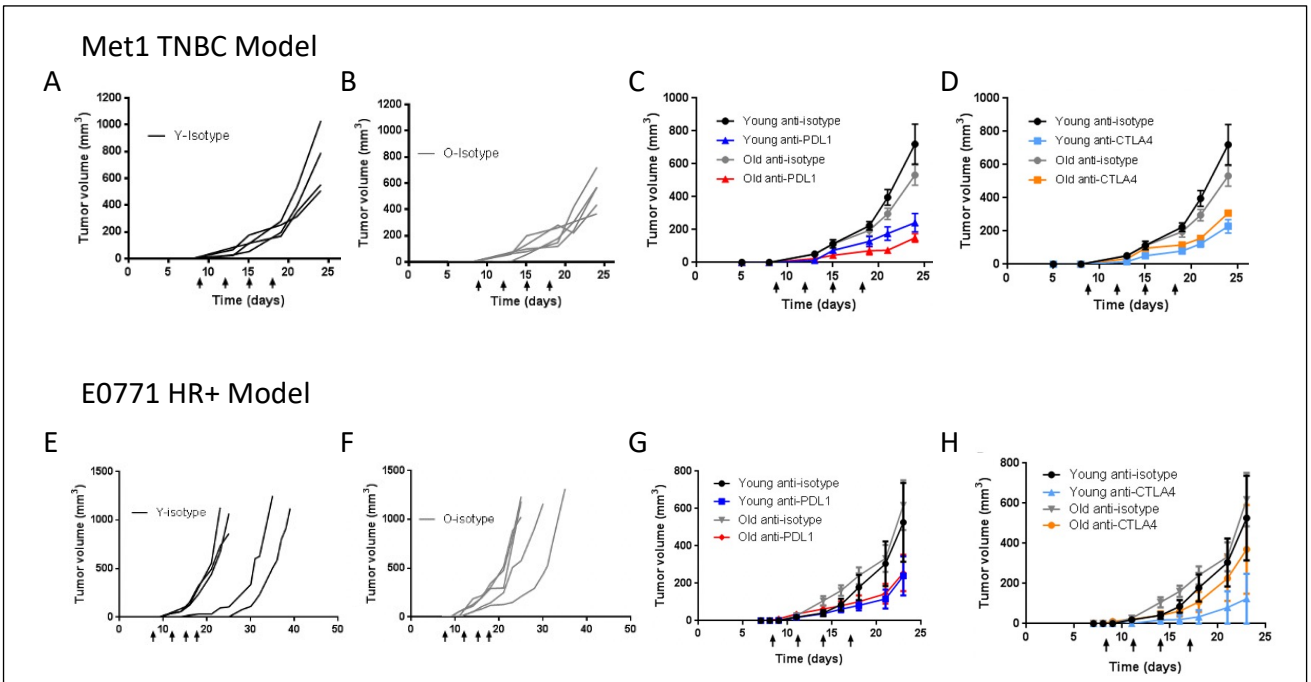


Figure 1. Age-dependent tumor growth and response to anti-PD-L1 in TNBC and HR+ pre-clinical models. (A,B) Met1 TNBC tumor growth kinetics in young (A) and old (B) isotype treated control mice. (**C,D**) Response to anti-PD-L1 (100µg, i.p.; C) and anti-CTLA-4 (200µg, i.p.; D) in young and aged cohorts of mice bearing Met1 TNBC tumors. (**E,F**) E0771 HR+ tumor growth kinetics in young (E) and old (F) isotype treated control mice. (**G,H**) Response to anti-PD-L1 (100µg, i.p.; G) and anti-CTLA4 (200µg, i.p.; H) in young and aged cohorts of mice bearing E0771 HR+ tumors.

We observed that the TNBC Met1 tumors grew more slowly in the aged mice than in the young mice (**Fig. 1A, B**), while HR+ E0771 tumor growth was similar in young and aged mice (**Fig. 1E, F**). It is interesting to note that those growth kinetics reflect the fact that TNBC is considered a more aggressive disease in young women, while HR+ disease is more prevalent in older women, suggesting these are good pre-clinical models. While all mice responded to anti-PD-L1 therapy, the magnitude of response was attenuated in the aged TNBC model (**Fig. 1C**) but not in the HR+ model (**Fig. 1G**). With respect to anti-CTLA4 treatment, the young TNBC mice had a greater magnitude of response to treatment than the old mice (**Fig. 1D**), while in the HR+ model, aged mice did not respond (**Fig. 1H**).

Second, we performed extensive immune-profiling by flow cytometric analysis of the tumors and blood in order to define age-stratified responses to tumor progression and ICB. Thusfar, we have been able to profile tumors and blood from the Met1 TNBC model. We observed both age-dependent and treatment-dependent changes in many of the major immune populations

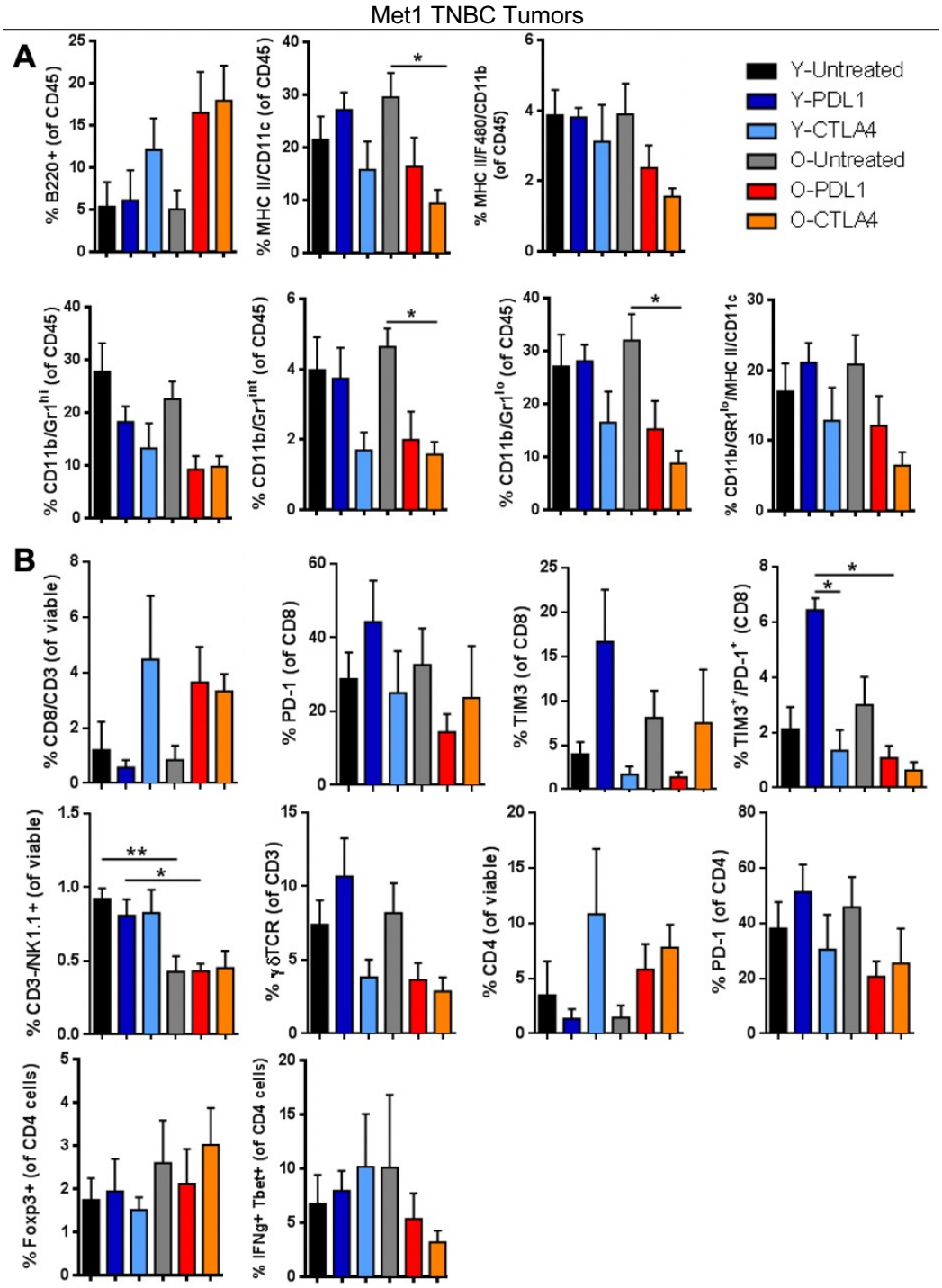


Figure 2. Immunoprofiling of the tumor microenvironment of Met1 TNBC tumors. End point tumors (from experiment described in Fig. 1) were evaluated for the indicated immune cell types/cell-surface markers by flow cytometric analysis. (**A**) Indicated cell types as a percentage of the total immune infiltrate (CD45+). (**B**) Indicated cell types as a percentage of the indicated specific immune

when compared with the young, untreated control mice (**Fig. 2**). In particular, we noted an increase in some of the exhaustion markers (e.g., TIM3+/PD-1+ CD8 T cells) in response to anti-PD-L1 only in the young mice (**Fig. 2B**), suggesting age-stratified effective anti-tumor immunity, which is in line with our observations of tumor responses to ICB (**Fig 1**).

We also analyzed intracellular hallmarks of effective immune responses in CD4+ and CD8+ T cells from the Met1 tumors. The most striking effects were observed with age; in the control mice, intracellular markers were significantly lower in the aged mice relative to the young mice,

suggesting that T cells in the aged mice were not able to be primed for anti-tumor immune responses (**Fig. 3**).

We also assessed the immunoprofile of the peripheral blood, in order to begin to define the first "immunologic age" profile in TNBC pre-clinical models. To do so, we used a hemacytometer to profile the peripheral blood at day 9 and day 18 from the TNBC tumor-bearing mice (experiment described in Fig. 1). The most striking observation was that lymphocyte counts were lower in the aged control mice relative to the young control mice and were not elevated with ICB as they were in the young mice (**Fig. 4**).

These immuno-profiling data were recently generated; therefore, we will continue our analyses. Our division has also recently acquired a Cytek Aurora full-spectrum flow cytometer, which will enable us to perform high dimensional single cell analyses. Thus, we have a great opportunity to further define the immune profiles of the tumor microenvironment and blood. We will also perform these analyses on our other breast cancer pre-clinical models to continue this line of investigation.

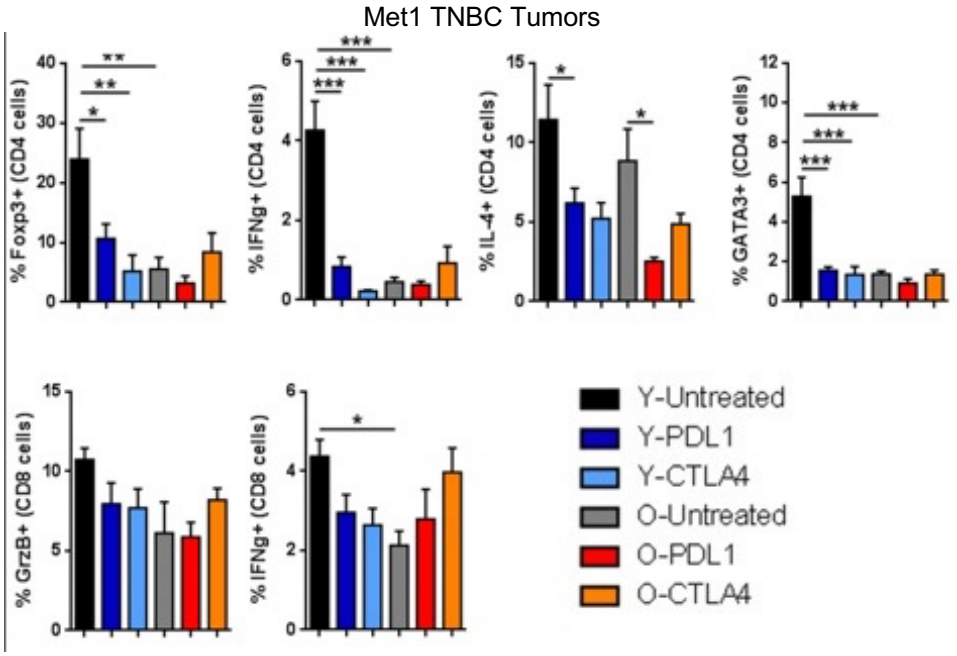


Figure 3. Evaluation of indicated intracellular factors and cytokines in indicated T cell subsets in Met1 tumors.

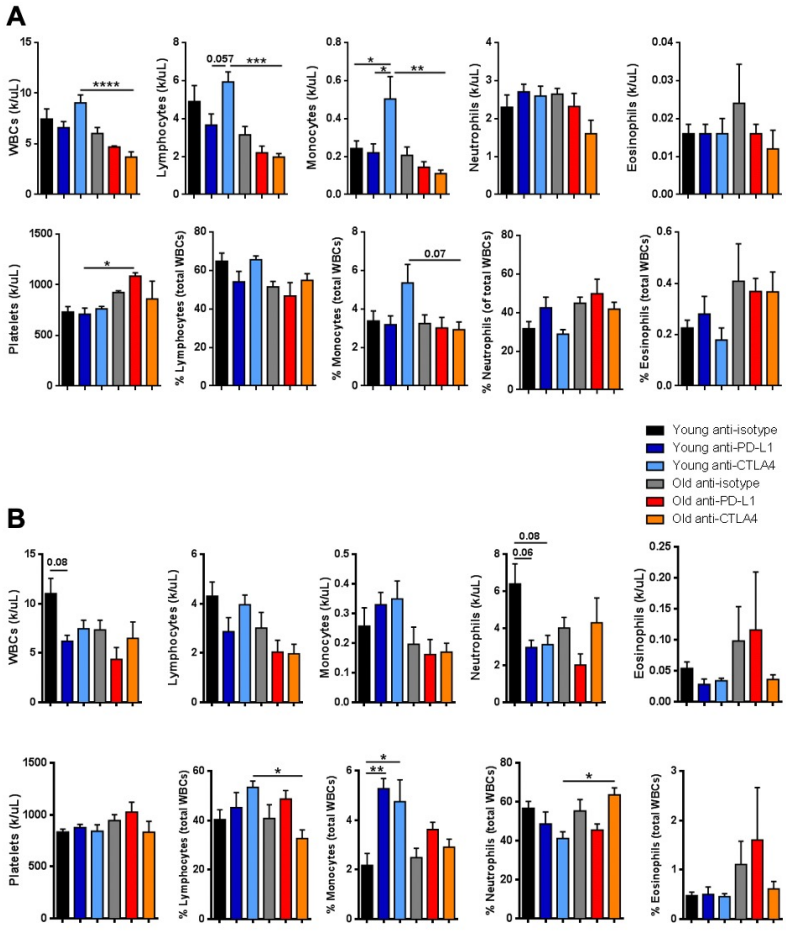


Figure 4. Analysis of peripheral blood from Met1 TNBC tumor-bearing mice. (A, B) Indicated blood counts as determined by hemacytometric analysis at day 9 (A) and day 18 (B) of tumor growth.

Another of our proposed goals associated with Major Task 1 is to perform RNAseq analysis to elucidate age-stratified gene expression signatures associated with response to ICB. We have generated these data and they are included below, as we performed those analyses in conjunction with our combination therapy experiments described under Major Task 3.

Major Task 2: Define Pre-Treatment Tumor Immunological Age in TNBC and HR+ Breast Cancer as a way to Predict ICB Response (proposed for months 12-30; 5% complete).

Due to the pandemic, patient enrollment and tissue collection for the ELEVATE trial (DFCI 18-634, older women with breast cancer) was delayed. Our clinical collaborators, Drs. Rachel Freedman and Ann Partridge have resumed enrollment/tissue collection and will provide us with de-identified archived FFPE tissues in this next funding period. We have also received our institutional IRB approval (secondary use) for this project and will submit our HRPO application with this progress report. Once we receive HRPO approval, we will be on track to perform the proposed analyses on these clinical tissues in months 14-32.

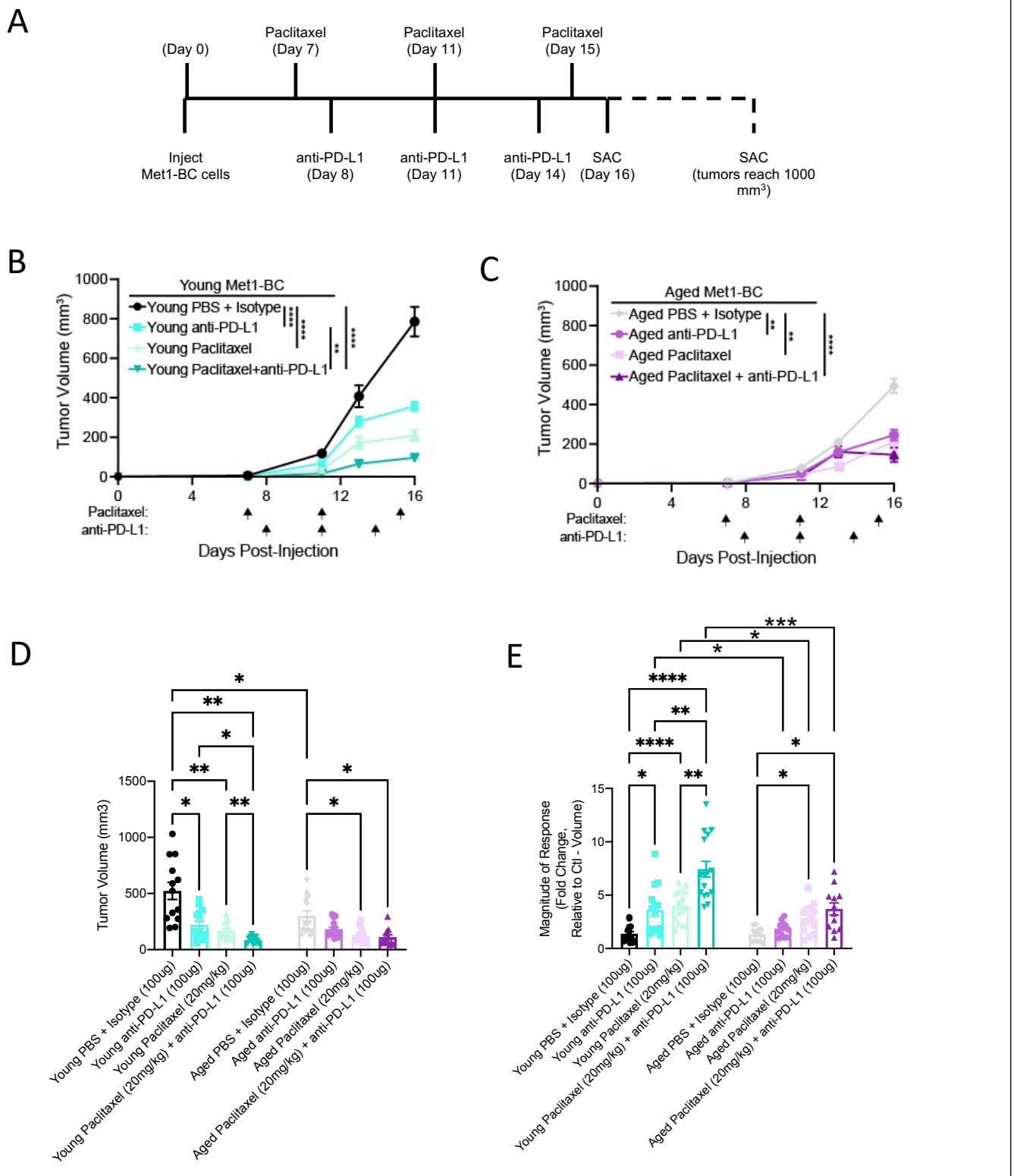
Major Task 3: Evaluate Efficacy of Pre-clinical ICB Combination Therapies in Young and Aged Cohorts. (proposed for months 1-20; 50% complete)

Currently, over 50% of breast cancer patients are older than 60 years of age at diagnosis; however, most clinical studies either exclude or under-enroll older patients. Likewise, young women are underrepresented in clinical trials. Therefore, while clinical trials evaluating ICB are now underway for breast cancer, those trials may be underpowered to determine if age impacts response to therapy. A critical consideration then, is whether real-world practice, which includes women of all ages, will reflect clinical trial observations, which are typically made in younger patients.

We therefore designed forward-thinking experiments to determine how young and aged mice respond to combination therapies that reflect some of the ongoing clinical trials that include ICB. During the first-year funding period, we focused on a pre-clinical trial to mimic abraxane (nabpaclitaxel) + atezolizumab (anti-PD-L1) in the neoadjuvant setting for TNBC (IMpassion 130 trial).

As shown in our original proposal, we injected $2.5x10^5$ Met1 cells orthotopically into cohorts of young and aged FVB mice. After 7 days, when tumors reached 2 mm in diameter, we randomly assigned treatment cohorts: vehicle + isotype controls, Paclitaxel, anti-PD-L1, or combination paclitaxel + anti-PD-L1. We administered 3 doses of paclitaxel (20mg/kg, I.P.) on days 7, 10 and 14. Mice in the ICB arms received 4 doses of either isotype (100ug) or anti-PD-L1 (100ug) on days 8, 11, 14, and 17. Primary tumor size was measured by digital calipers beginning at day 7 and every 3 days throughout the experiment (**Fig. 5A**).

We found that both young and aged mice responded to pax with a reduction in tumor growth during the course of treatment (**Fig. 5B, C**). The young mice also showed significant reductions in tumor growth in response to anti-PD-L1 monotherapy and the combination therapy (**Fig. 5B, D**). However, the aged mice responded to monotherapy, but did not experience additional benefit of the combination therapy (**Fig. 5C, D**). In fact, when we plotted the magnitude of response relative to age-matched controls, only young mice saw benefit from addition of anti-PD-L1 to paclitaxel (i.e., the clinically relevant scenario) (**Fig. 5E**).



F**igure 5. Pre-clinical evaluation of combination paclitaxel +/- anti-PD-L1 in age-stratified TNBC**. (**A**) Schematic of treatment regimen. (**B**) Response to monotherapy and combination therapy in young FVB mice with Met1 TNBC. (**C**) Response to monotherapy and combination therapy in aged FVB mice with Met1 TNBC. (**D**) Tumor volumes at experimental end point (day 16). (**E**) Magnitude of responses relative to age-matched isotype control mice.

We performed RNAseq analysis on the resulting tumors from these cohorts (Fig. 5). PCA analysis revealed clear differences between young and aged mice for all treatment cohorts (**Fig. 6A**), suggesting age-dependent effects on tumor growth (isotype controls) as well as treatment. An initial GO term analysis comparing isotype-treated young and aged mice revealed significant differences in immune pathways (**Fig. 6B**). These data reinforce and build on our findings that immunologic age is a powerful and dominant factor in determining disease progression and response to ICB. We are currently in the process of continuing to analyze all of the RNAseq data in our efforts to uncover the first "immunologic age" signatures.

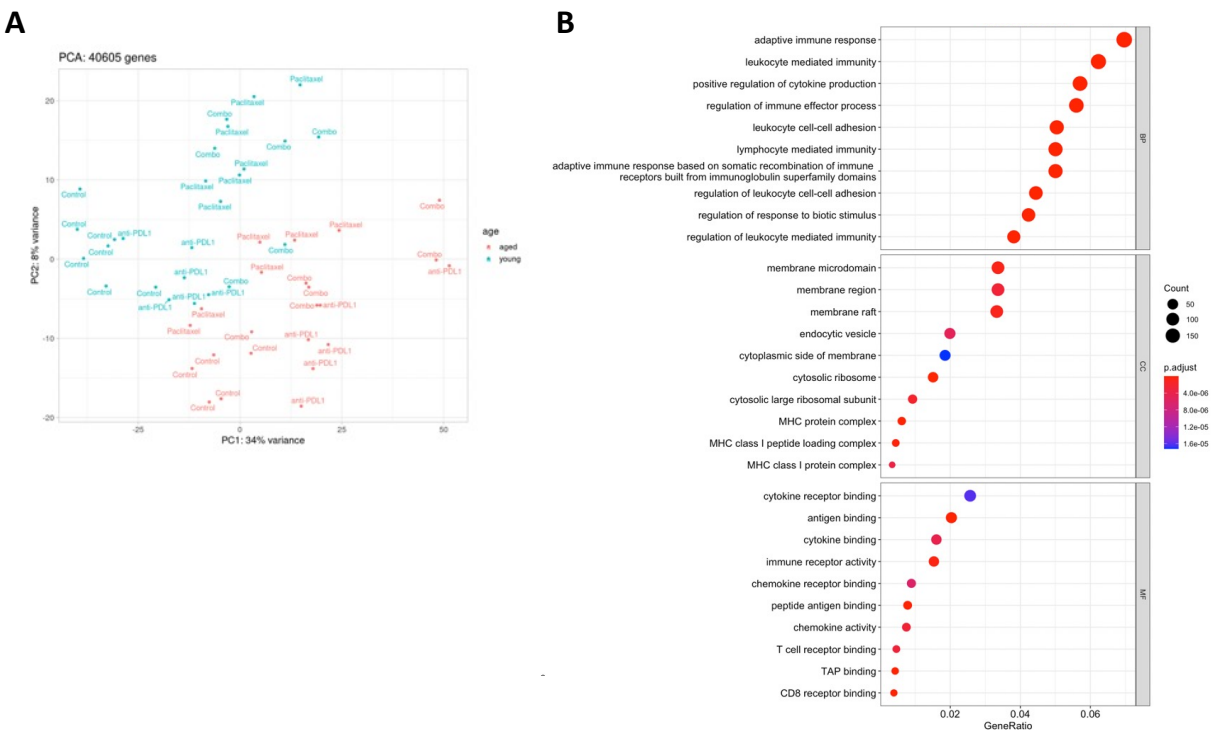


Figure 6. RNAseq analysis on young and aged mice treated with paclitaxel or anti-PD-L1 monotherapy, or combination paclitaxel + anti-PD-L1. (**A**) PCA analysis of RNAseq data. (**B**) GO terms ranked by count and padj values comparing young to aged isotype control mice.

- **What opportunities for training and professional development has the project provided?**

  Dr. Milos Spasic, postdoctoral fellow, who is performing these studies, had an opportunity to attend and present a poster at the AACR Annual meeting. We will continue to seek opportunities for him to attend seminars and conferences as the work matures.

- **How were the results disseminated to communities of interest?**

  In March of this year, we met with the DFCI Patient and Research Advocates to present our results and discuss their implications. We also discussed future experimental design and connecting with the clinical team.

- **What do you plan to do during the next reporting period to accomplish the goals?**

  Briefly, we intend to focus on the following work during the next reporting period:

  Task 1:

  - Continue to perform immunoprofiling of pre-clinical models via high-dimensional full spectrum flow cytometry
  - Continue with data analyses

Task 2:

- Obtrain DOD HRPO approval
- Obtain FFPE tissues from the clinical trials (young and older breast cancer patients) and perform multi-plex staining and analysis for proposed age-dependent immune hallmarks.
- Potential add-on approach: We may also be in a position to obtain blood samples from these same patients, in which case, we can perform high-dimensional flow cytometry in efforts to define “immunologic age” in blood samples from breast cancer patients.

Task 3:

- Continue with computational analyses of RNAseq data from combination therapy trial
- Begin to validate some of the immune markers as proposed.
-

4. IMPACT:

Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

6. PRODUCTS:

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

No changes

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES:

None