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TITLE: Overcoming Immunotherapy Resistance in Breast Cancer Using RT-Mediated Immunomodulation

PRINCIPAL INVESTIGATOR: Simon Knott, Stephen Shiao

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
8700 Beverly Blvd
Los Angeles, CA, 90048

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14. ABSTRACT

Breast cancer generally exhibits low response rates to immunotherapy such as PD-1/PD-L1 blockade for multiple reasons including tumor-mediated immune suppression and poor immunogenicity. We sought to change the responsiveness of breast tumors to anti-PD-1 therapy by reshaping the tumor immune microenvironment using focal radiation (RT). Irradiation of breast tumors elicits direct killing of malignant cells, but more importantly generates an anti-tumor immune response. This immune reaction is a critical mediator of both primary tumor control and abscopal responses, though the cellular and molecular dynamics within the immune compartment following treatment are incompletely understood. We elucidated these changes using single-cell sequencing in a preclinical model of breast cancer. We found that immediately following RT intratumoral immune cells are largely eliminated and a de novo inflammatory response initiated. The ensuing response consists of activated cytotoxic CD8+ T cells and CD11b+ myeloid/macrophages, but also induced regulatory T cells and inhibitory signals including PD-1 on T cells and PD-L1 on macrophages. We found that RT synergizes with PD-1 blockade through its ability to alleviate intratumoral immunosuppression and stimulate the production of cytotoxic T cells through the generation of a hyperphagocytic, cytotoxic and antigen presenting macrophage phenotype. Examination of biopsies from breast cancer patients undergoing neoadjuvant anti-PD-1 and RT revealed similar changes. We observed enhanced macrophage mediated killing and antigen presentation leading to increased numbers and activation of intratumoral immune responses across more than 80% of patients examined. These observations support the notion that RT mediated changes in the tumor immune microenvironment enhance the sensitivity of breast tumors to checkpoint blockade.

15. SUBJECT TERMS

Breast cancer, Single cell sequencing (SCseq), Immunotherapy, Radiation Therapy (RT), Macrophage, CD8+ T cell, CD4+ T cell, B cell

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Most breast cancers remain resistant to immunotherapy in part through the establishment of an immunosuppressive microenvironment by malignant cells. Radiation therapy (RT) can dramatically alter the immune microenvironment of tumors both through elimination of the existing immune cells within tumors and stimulation of a de novo immune response. Thus, *our overall hypothesis is that combining focal breast RT with immunotherapy will increase the efficacy of immunotherapy for breast cancer by favorably reprogramming the tumor immune microenvironment of breast tumors.* Given the resistance of most breast cancers to immunotherapy and the ability of RT to alter the tumor immune microenvironment, we proposed three aims to understand and target the mechanisms by which RT shapes the immune microenvironment of breast tumors to augment antitumor immune responses. In the first two aims we proposed to characterize the local and systemic immune responses to RT and anti-PD1 a murine model of breast cancer, and then to use the same model and knockout mouse lines lacking different immune cells to elucidate the mechanisms by which RT improves immunotherapy responses. In a final aim we proposed to examine the role of RT in altering the response to anti-PD1 therapy in a human breast cancer trial of neoadjuvant RT and immunotherapy

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Breast cancer, Single cell sequencing (SCseq), Immunotherapy, Radiation Therapy (RT), Macrophage, CD8+ T cell, CD4+ T cell, B cell

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

The three main goals for this project are (1) Characterize the impact of RT-induced changes in the tumor immune microenvironment on the efficacy of immunotherapy both locally and systemically, (2) Define mechanism(s) of RT-mediated modulation of tumor responses to immunotherapy and (3) Examine the role of RT in modulating the efficacy of immunotherapy in a human breast cancer trial of neoadjuvant RT and immunotherapy.

What was accomplished under these goals?

For the reporting period Months 1-3, we obtained IACUC approval for the proposed murine studies, hired and trained staff for the mouse experiments (Shiao) and downstream processing of tumor samples by single cell RNA sequencing (Knott). Finally, we also submitted and acquired local IRB approval for the proposed clinical trial (Shiao).

For the reporting period Months 4-9, we received full approval from ACURO and HRPO to begin our murine experiments and clinical trial (Completion of Milestone 1A and 1B). There was been some delay due to laboratory shut downs related to COVID19 however research activities resumed and Specific Aim 1, Major Task 1, Subtask 4 began. The clinical trial also began enrolling patients for the Phase Ib portion of the trial with biopsies being collected and processed for single-cell sequencing (Specific Aim 3, Major Task 1, Subtasks 3 and 4).

For the reporting period Months 9-12, we performed single tumor single dose RT and anti-PD1 experiments in mice Aim 1 and Subtask 2 and corresponding single cell sequencing analysis. Due to COVID we were slightly delayed on the multidose experiments (Aim 1 Subtask 2), but preliminary experiments have now been performed and these tasks will be completed imminently. We have also completed enrolling patients in the phase Ib trial (Aim 3 Major Task 1), with all biopsies

being successfully analyzed from these patients as well as the first two patients from the phase II trial (Aim 3, Major Task 2).

Each of the detailed goals to be completed up to and including this reporting period are noted in the table below. Following the table is a detailed description of the scientific findings from these studies.

Specific Aim 1: Characterize the impact of RT-induced changes in the tumor immune microenvironment on the efficacy of immunotherapy both locally and systemically.	**Timeline (Months)**	**CSMC (Stephen Shiao, MD, PhD)**	**CSMC (Simon Knott, PhD)**	**Status**
Major Task 1: Effects of different doses of RT on the efficacy of PD-1/PD-L1-directed immunotherapy				
Subtask 1: Obtain local IACUC approval				
Obtain local IACUC approval	0	Dr. Shiao		**Done**
Subtask 2: Train new staff				
Train new staff for animal experiments	1-3	Dr. Shiao		**Done**
Train new staff for tumor dissociation, cell isolation and NGS library construction	1-3		Dr. Knott	**Done**
Subtask 3: Single tumor, single dose radiation study with anti-PD-1				
Expose tumor-bearing mice (20/group) to a single-dose of low, medium and high dose radiation & monitor tumor growth & collect blood samples/fecal pellets until indicated time points for tumor tissue analysis (endpoint, 3, 7 and 10 days post-radiation). (160 animals)	4-7	Dr. Shiao		**Done**
scSeq library preparation, quality control (QC), pooling and sequencing	7		Dr. Knott	**Done**
scSeq data analysis	8-9		Dr. Knott	**Done**
Subtask 4: Single tumor, multidose dose radiation study with anti-PD-1				
Expose tumor-bearing mice (20/group) to a multi-dose course of low, medium and high dose radiation & monitor tumor growth & collect blood samples/fecal pellets until indicated time points for tumor tissue analysis (endpoint, 3, 7 and 10 days post-radiation). (160 animals)	8-11	Dr. Shiao		**In Process**
scSeq library preparation, quality control (QC), pooling and sequencing	11		Dr. Knott	**Awaiting Samples**
scSeq data analysis	12-13		Dr. Knott	**Awaiting Samples**
Milestone Achieved: ACURO Approval	4	Dr. Shiao		**Done**

Specific Aim 3: Examine the role of RT in modulating the efficacy of immunotherapy in a human breast cancer trial of neoadjuvant RT and immunotherapy	**Timeline**	**Site 1 (Stephen Shiao, MD, PhD)**	**Site 2 (Simon Knott, PhD)**	**Status**
Major Task 1: Conduct initial phase Ib trial to assess the safety of neoadjuvant RT and immunotherapy				
Subtask 1: Submit documents for local IRB review and approval				
Submit documents for local IRB review and approval	1-4	Dr. Shiao		**Done**
Subtask 2: Submit IRB approval and necessary documents for HRPO Review				
Submit IRB approval and necessary documents for HRPO Review	4-7	Dr. Shiao		**Done**
Subtask 3: Enroll first 10 patients on the phase Ib				
Enroll first 10 patients on the phase Ib (Key Collaborators: Dr. McArthur, Basho, Giuliano and Karlan)	7-13	Dr. Shiao		**Done**
Subtask 4: scSeq analysis of clinical trial biopsies				
scSeq library preparation, QC, pooling and sequencing	7-13		Dr. Knott	**Done**

Murine Studies

Analyses of previously acquired data

To elucidate how RT changes the intratumoral leukocyte population following radiation therapy (RT), we previously applied single cell RNA sequencing (scSeq) to the immune cells of orthotopic E0771 murine mammary tumors following a single fraction of focal irradiation (16 Gray, Gy). The beam arrangement and dose selected ensured the treatment encompassed the entire tumor while avoiding surrounding tissue, and that RT delayed tumor growth and prolonged survival (Fig 1a). $CD45^+$ cell populations from tumors that were irradiated 3, 7, 10- and 14-days prior at sizes of ~1 cm^3 were collected and analyzed.

As previously described[1], we found that the majority of immune cells at baseline were T cells and macrophages, though others including NK cells, B cells and Dendritic Cells (DC) were also present (Fig 1a). Following irradiation, we observed a dramatic reshaping within both the T cell and macrophage compartments, with the elimination of most baseline populations completely at Day 3 followed by the appearance of new populations starting at Day 3 and continuing through Day 10 (Fig. 1a). Interestingly, the original immune cell repertoire was largely restored by Day 14.

To examine the functional state of the CD8+ T cells (CD8T) following RT, we used established markers to classify constituents as naïve, activated, exhausted or induced suppressive and then tracked their proportions within the tumor over time (Fig. 1b). Naïve cells were low for CD44 and CD69 and high for CD62L, and an analysis of transcription factor activity scores suggested this program was driven by Tcf7 (Fig. 1c). Activated and exhausted cells both expressed a Runx3 driven activation program corresponding to diffusion component 1, that harbored the genes Granzyme B, perforin, CD69 (Fig. 1c-d). Differentiating these groups were Lag3- and Tim3-expression in the exhausted cells and a proliferative signature in the activated group, corresponding to diffusion component 3 (Fig. 1d). Overall, the naïve T cell population expanded 1.5x by day 3 after RT, while the activated population dominated Days 7 and 10. The activation signature wanes by day 14 with the cellular and molecular signatures returning to baseline. This timeline mirrors other cytotoxic T

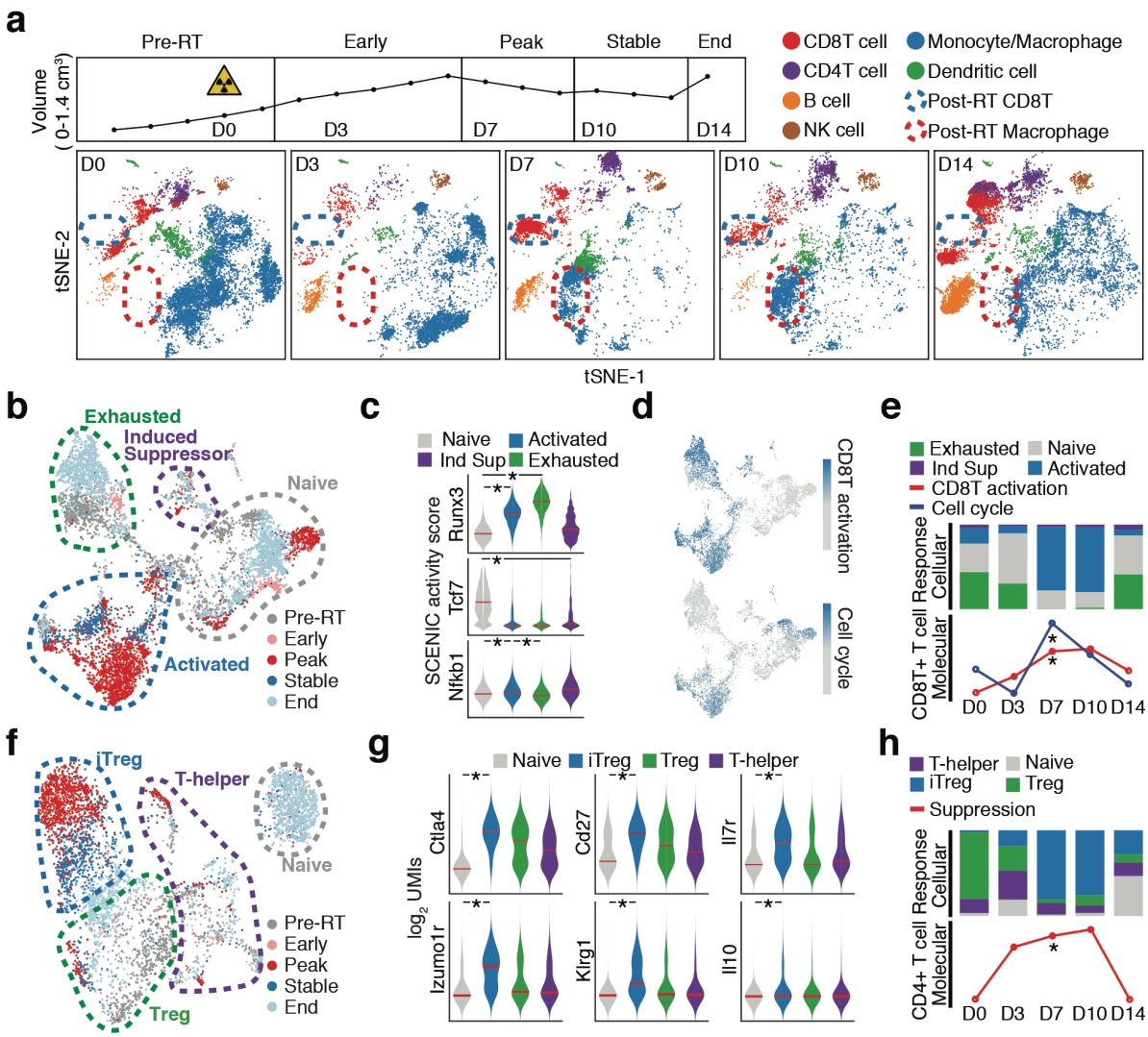

Figure 1. Radiation therapy perturbs tumor immune microenvironment through the activation of CD8 T-cells with a compensatory CD4 regulatory T-cell response. (a), Representative radiation-treated orthotopic E0771 tumor growth curve (top) and corresponding tSNE embedding of the tumor immune microenvironment at indicated time points (bottom). Cells are colored by cell type and dashed lines highlight RT induced changes in the innate and adaptive arms. **(b),** UMAP embedding of CD8 T-cells (CD8T) from **a**. **(c),** Transcription factors differentially activated in the CD8T population, as predicted by SCENIC ($p < 10^{-15}$). **(d),** Diffusion component (DC) scores corresponding to CD8T activation and cell cycle overlaid on UMAP embedding from **d**. **(e),** Composition of CD8T subpopulations, as annotated in **d**, (top) and average DC score of CD8Ts, as shown in **d**, (bottom) across timepoints ($p < 10^{-100}$ D7 vs D0). **(f),** UMAP embedding of intra-tumoral CD4 T-cells (CD4T). **(g),** Key genes differentially expressed in induced regulatory T-cells (iTreg) (FDR $< 10^{-15}$). **(h),** Composition of CD4T subpopulations, as annotated in **g**, (top) and average DC score of CD4Ts (bottom) across timepoints ($p < 10^{-95}$ D7 vs D0).

cell responses such as viral infections indicating that RT triggers a response akin to a de novo immune response in tumors[2].

A similar analysis of CD4+ T cells (CD4T) identified naïve, T-helper, regulatory T cells (Tregs), but the most interesting change was the appearance of an “induced” Treg (iTreg) population that strongly peaks through Days 7 and 10 (Fig. 1f-h). iTregs express a unique suppressive signature harboring regulatory genes such as IL-10, Izumo1r, CD27 and Klrg1, which is defined by the first diffusion component of the $CD4^+$ population and is similar to that of TGFβ1-induced regulatory T

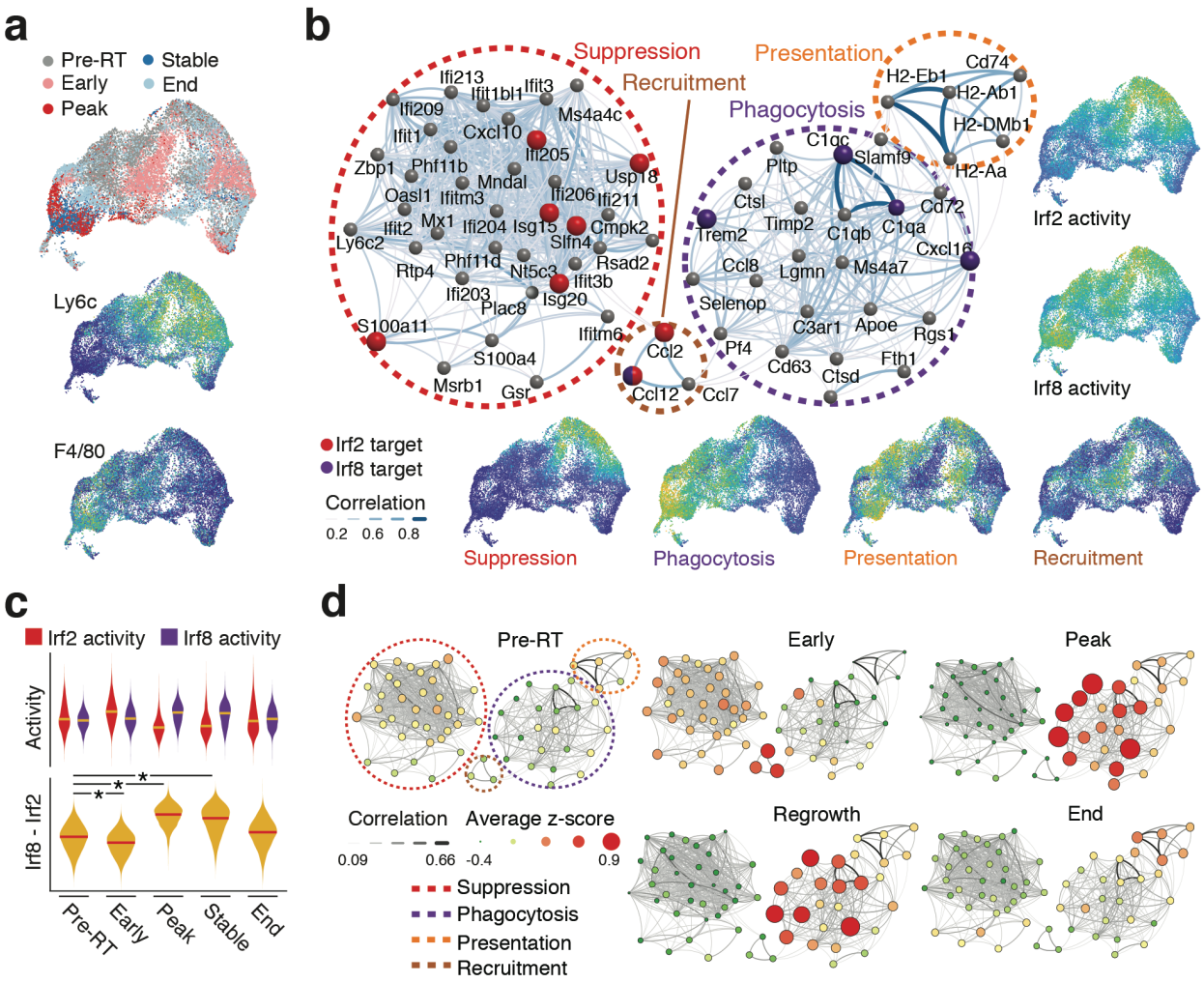

Figure 2. Radiation therapy induces an acute transition of intra-tumoral macrophages from a suppressive to a phagocytic phenotype. (a), UMAP embedding of intra-tumoral macrophages colored by time point (top) and expression of key phenotypic markers overlaid (bottom). **(b),** Gene network correlation graph derived de-novo from macrophage data with core modules highlighted, weight of edges determined by inter-gene correlation, and nodes colored if predicted to be downstream targets of Irf2 or Irf8, as determined by SCENIC regulons. The network graph is surrounded by overlays of median core module expression and Irf2 and Irf8 SCENIC activity scores on the UMAP from **a**. **(c),** Irf2 and Irf8 activation, as predicted by SCENIC, and difference in activation status in macrophages across timepoints ($p < 2.9x10^{-146}$). **(d),** The same network graph as in **b**, but with node size and color determined by the average z-scored expression of the node at each time point.

cells described previously[3]. Our findings support the notion that the strong inflammatory response induced by RT drives a compensatory regulatory response, which other groups have shown is a critical mediator of local and systemic anti-tumor immunity following RT[4].

We next examined monocyte/macrophage cells using a gene co-expression analysis and identified 4 major expression modules. These consisted of Ly6c^{-hi}F4/80^{lo} suppressive and Ly6c^{lo}F4/80^{hi} phagocytic modules with intermediate Ccl2^{hi} recruitment and MHC-Class II^{hi} presentation modules (Fig. 2a & b). Additionally, we identified Irf2 and Irf8 as potential regulators of the suppressive and phagocytic modules, respectively (Fig. 2b & Extended Data Fig. 4b). Irf2 expression in macrophages drives an anti-inflammatory program[5] whereas Irf8 drives anti-tumor immunity[6]. In comparison to baseline, macrophages were most significantly altered in their expression program after 7 and 10 days. Following RT, an Irf2^{lo}Irf8^{hi} phagocytic population overtook the compartment and remained beyond Day 10 (Fig. 2c & d). However, Irf2 and Irf8 levels rebalanced

by Day 14, returning module expression to baseline levels. Our findings indicate RT initiates a transient reprogramming of macrophages from an immunosuppressive, homeostatic phenotype to a cytotoxic, antigen-presenting phenotype. The reversion to baseline is likely driven by the suppressive signals produced within macrophages consuming apoptotic cells[7].

To better understand the activation pathways governing this RT-mediated transformation we performed a focused examination of known checkpoint proteins and found that RT upregulated PD-1 in CD8Ts, PD-L1 in CD4Ts and PD-L1 and SIRPA in macrophages (Fig. 3a). This data and previous studies in melanoma and NSCLC showing synergy between RT and immune checkpoint blockade prompted us to ask if targeting the PD1/PD-L1 axis in the context of RT could promote further immune mediated killing in our murine model of breast cancer[8,9].

Acquisition and Analysis of Murine Data for Current Project

As part of Aim 1 of this current application, we enrolled orthotopic tumor bearing mice into each of four study arms, which included IgG control antibody, anti-PD1 antibody, IgG control antibody with RT and anti-PD1 antibody with RT. Control tumors grew quickly, with all mice requiring euthanasia by Day 45 (Fig 3b). Mice receiving RT or anti-PD1 alone saw tumor shrinkage followed by regrowth. In contrast, animals administered the combination showed complete tumor regression and 100% survival beyond 90 days.

To study the changes in the immune compartment underlying this synergism, we extracted tumors on Day 5 following RT and where three doses of PD1 blocking antibody had been administered and analyzed leukocytes with scSeq. As was observed previously, RT alone increased the proportion of activated $CD8^+$ T cells, which was not seen with PD1 blockade, despite the associated increased survival (Fig. 3e). Instead, PD1 blockade expanded the exhausted CD8T population at the expense of naïve T cells. Combination therapy boosted activated CD8Ts compared to RT alone with a corresponding decrease in naïve CD8Ts. Interestingly, RT uniquely induced an iTreg population and the combination treatment drove an expansion in this group that was five times greater than with RT alone. Our studies show that combining RT with anti-PD1 augments the numbers and function of cytotoxic T cells similar to other preclinical breast models that have demonstrated synergy with this combination[10]. However, we also show there is a simultaneous increase in a regulatory T cell population, which likely governs the ability of RT to induce an abscopal response beyond the irradiated tumor.

RT again also uniquely drove expansion of $F4/80^{hi}$ and $Ly6c^{lo}$ macrophages, and a gene co-expression analysis determined the phagocytic and presentation modules were activated in this population (Fig 3d). PD1 blockade produced a similar phenotype, but with tempered phagocytic and recruitment module activity and slightly higher presentation gene levels. When combined, RT and PD1 blockade collaborated for a specific expansion (20%) of the RT-associated macrophage population with elevated phagocytic and antigen presenting capacity (Fig. 3d). This activity likely drives the enhanced cytotoxic response following RT as tumor-associated macrophages are important potential drivers of anti-tumor immunity[11].

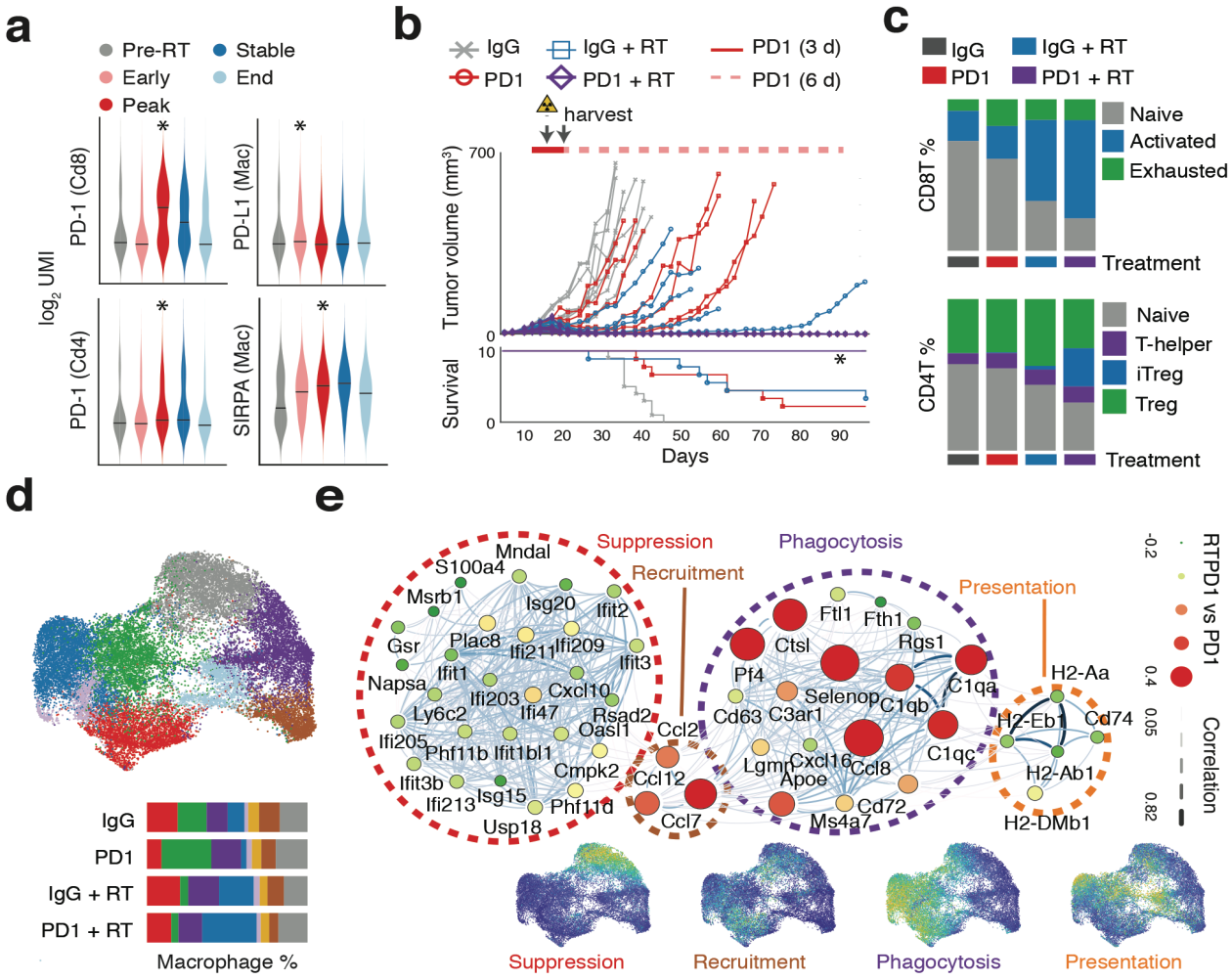

Figure 3. Combination anti-PD-1 and radiation treatment induces durable anti-tumor response characterized by a robust CD8 T-cell activation and macrophage phagocytic/presentation phenotype. (a), Expression of key immune checkpoint molecules in CD8 and CD4 T-cells (CD8T and CD4T) and macrophages (Mac) following radiation therapy. (PD-1 CD8T D7 vs D0 FDR = $1.2E^{-50}$, PD-1 CD4T D7 vs D0 FDR = $1.1x10^{-16}$, PD-L1 Macs D3 vs D0 FDR = $3.9x10^{-193}$, SIRPA Macs D7 vs D0 FDR = $5.5x10^{-132}$). **(b),** Individual tumor growth curves (top) and survival curve (bottom) for orthotopic E0771 tumor bearing mice administered control antibody (IgG), anti-PD-1 (PD1), IgG and radiation (IgG+RT), or PD1 and RT (PD1+RT) ($p = 2.2x10^{-8}$). **(c)**, Composition of intra-tumoral CD8T (top) and CD4T (bottom) subpopulations in indicated treatment groups. **(d),** UMAP embedding of macrophages from all samples combined and colored by cluster (top) accompanied by the composition of macrophage clusters in indicated treatment groups colored as in UMAP (bottom). **(e),** Macrophage gene network correlation graph with core modules highlighted, weight of edges determined by inter-gene correlation, and nodes sized/colored by the average z-scored (across macrophages in PD1+RT and PD1 groups) expression of the node (top). Expression of each core module overlaid on the UMAP from **d** (bottom). Data in **c, d,** and **e** are from tumors harvested where indicated in **b**, i.e. 5 days after radiation treatment, where two independent cohorts of mice were analyzed, combining two tumors from each treatment group for each cohort.

SCseq Analysis of Immune Cells from Phase IB and II Clinical Trial

As listed in Aim 3 of our SOW, we executed a phase II clinical trial to study the anti-PD1 antibody pembrolizumab in combination with RT in the neoadjuvant setting for women with triple-negative breast cancer (TNBC). Additionally, we have enrolled one patient into our phase II trial Aim 3 Major Task 2, and have analyzed these samples here as well. In total, 11 newly diagnosed TNBC patients received pre-operative pembrolizumab, followed three weeks later by pembrolizumab with RT (24 Gy/3 fractions) to the primary breast tumor only, which was then followed by standard-of-care chemotherapy, surgery and RT (Fig. 4a). Of the 11 patients evaluated, 8 (82%) showed a

pathological pathologic complete response at the time of surgery and three of the four baseline node-positive patients were cleared of metastases, demonstrating the potential of the combination.

We examined the immune compartment changes brought on by anti-PD1 and anti-PD1 with RT by performing scSeq on $CD45^+$ cells obtained from research biopsies taken from each patient at baseline, 3 weeks after pembrolizumab and 3 weeks after combined RT and pembrolizumab. In total we analyzed 135,041 immune cells, with 51,074 coming from pre-treatment biopsies, 35,224 coming from post-pembrolizumab biopsies and 48,743 from biopsies extracted after the combination therapy. As in orthotopic EO771 tumors, macrophages, CD8+ and CD4+ T cells were found to be in high abundance in most human TNBC tumors, and the variation within these populations appeared driven by many of the same programs as were described in murine cells (Fig. 4b). However, inter-tumor heterogeneity was also found to be a major source of variation.

To analyze human macrophages, we averaged pairwise gene correlations from each patient tumor and applied thresholding to develop a co-expression network (Fig. 4c). This identified a three-module network bearing strong resemblance to the murine transcriptional system. One module overlapped with the murine suppressive subnetwork, but for a lack of interferon response genes. Furthermore, MHC class II genes, including murine orthologs CD74 and HLA-DQB1, were harbored in a second component. The remaining subnetwork overlapped strongly with the murine phagocytic module, with both harboring the same tightly correlated complement pathway gene set. The lack of the recruitment subnetwork may reflect the later timepoint of the biopsy compared to the preclinical model, since this subnetwork was most evident at Day 3 following RT.

Following classification based on the expression of these modules, we followed intratumoral macrophage proportions and found pembrolizumab induced moderate changes, including increased phagocytic and decreased suppressive cell proportions (Fig. 4d). Combination therapy significantly amplified this shift to a phagocytic phenotype compared to anti-PD-1 alone. Additionally, the antigen presenting population was increased following administration of the combination therapy. When comparing global gene expression differences, we found the baseline population exhibited a suppressive phenotype, whereas pembrolizumab treated tumors harbored more of the phagocytic macrophage phenotypes. In contrast, macrophages from RT and pembrolizumab treated tumors maximally express the complement and antigen presentation transcriptional modules. DCs also exhibited elevated TLR4 signaling and MHC Class I processing in mouse and human tumors following RT, consistent with enhanced activation (data not shown).

Within the CD4T population we found seven cell phenotypes, which were unchanged in proportion by either the single or combination treatment. However, RT induced expression of many regulatory genes in human Tregs where corresponding orthologs were also upregulated in murine iTregs. Expanded CD4Ts were reduced in naïve and early activated phenotypes and increased in Th2 regulatory cell proportions. However, treatment did not induce expansion of CD4Ts.

Naïve CD8Ts were moderately increased in proportion following pembrolizumab, and memory cells were slightly enriched after the combination therapy (Fig. 4e). However, an advanced activation CD8 T-cell phenotype increased following combination treatment. This phenotype comprised both activation (Perforin, GZMB) and exhaustion (LAG3, TIM3) genes along with previously described tumor-reactive markers such as CD103 and CXCL13. Transition probabilities calculated on TCRs present in the last two biopsies revealed RT could stimulate pre-existing clonotypes to display this signature regardless of their prior subtype distribution. However, many newly expanded TCRs were also detected after RT, driving an increase in the overall proportion of expanded cells in the CD8T population (Fig. 4f). Most importantly, these newly established clonotypes, like the expanded set in general, were found to be enriched for cells of the advanced activation subtype (Fig 4g).

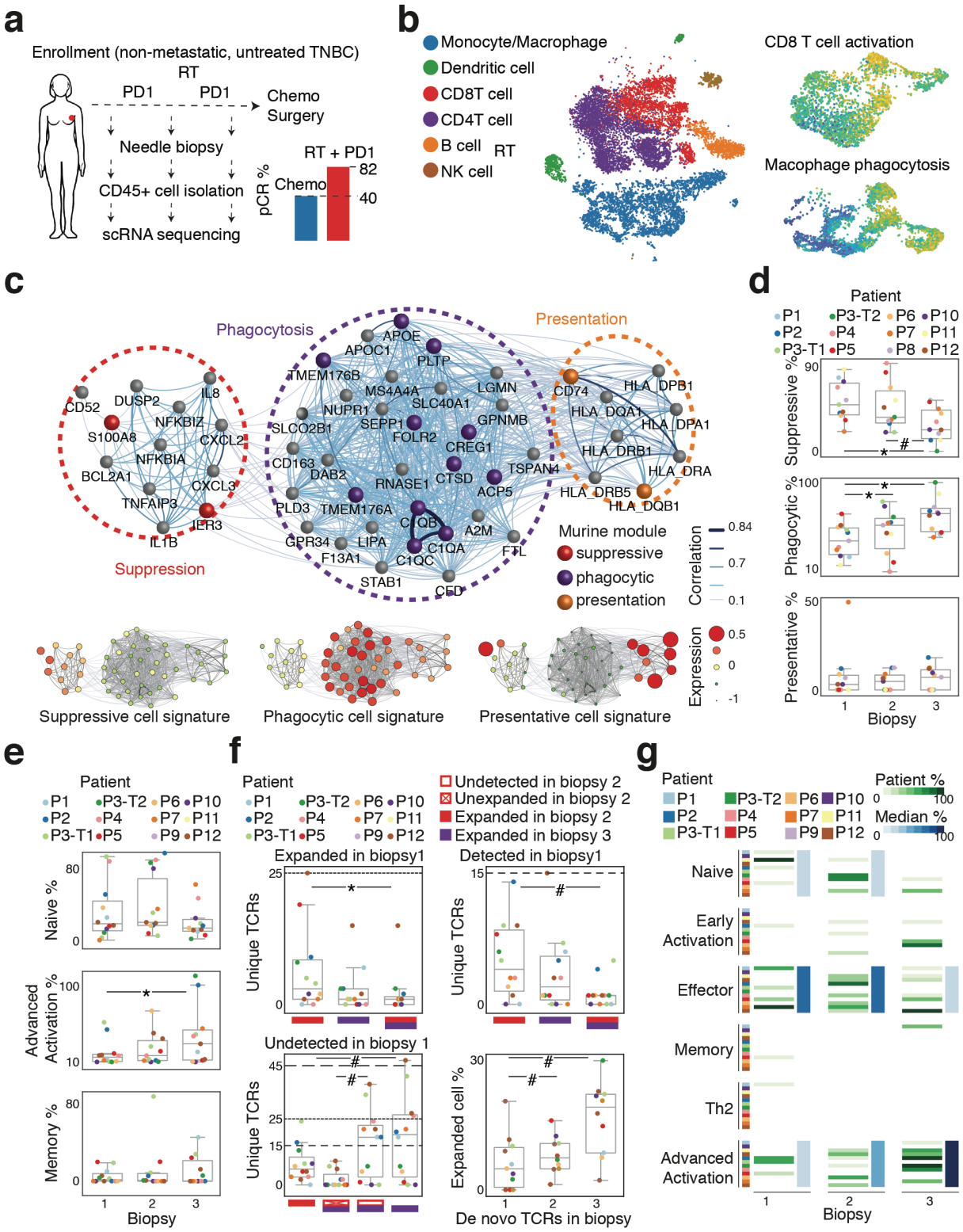

Figure 4. Neoadjuvant anti-PD-1 and radiation treatment induces macrophage phagocytic/presentation phenotype and unique CD8 T-cell clonal expansion characterized by an advanced activation signature in human triple-negative breast tumors.

Figure 4 legend continued. (a), Design of clinical trial and biopsy schedule with comparison of pathological complete response rates (pCR) from neoadjuvant chemotherapy (Chemo) alone (Cortazar et al., Meta-Analysis, Lancet, 2014) and current trial results (RT+PD1). **(b),** tSNE embedding of $CD45^+$ cells from a human triple negative breast tumor (left), and CD8 T-cell (CD8T) activation and macrophage phagocytosis module scores overlaid on UMAP embeddings of the respective cell types (right) from the same patient. **(c),** Gene co-expression network derived de-novo from patient intra-tumoral macrophage data with core modules highlighted, edge weights determined by inter-gene correlation, and nodes colored if they were identified in the respective mouse macrophage network core module (top). The same network graph with nodes sized/colored according to expression levels in each macrophage subtype (suppressive, phagocytic, or presentative) are shown below. **(d),** Subtype composition of total macrophages in each biopsy. Dots represent individual patients ($p < 0.05$ and # $p \leq 0.01$). **(e),** Phenotypic composition of CD8Ts in each biopsy where dots represent individual patients (t-test, Naïve RTPD vs Base $p < 0.1$, * $p < 0.05$). **(f),** Numbers of unique CD8T T-cell receptors (TCRs) that satisfy the indicated criteria (all except bottom right) and the percentage of expanded CD8Ts in clonotypes that are unique to each biopsy (bottom right), each dot represents an individual patient. ($p < 0.05$ and # $p \leq 0.01$). **(g),** Phenotypic composition of CD8Ts possessing TCRs that satisfy the criteria of **f** (bottom right) separated by patient (rows in green) and the median patient values (columns in blue) across biopsies.

Summary of Results

Our early studies demonstrate that RT can alter the immune microenvironment of breast tumors in a characteristic pattern and that adding RT to anti-PD-1 therapy leads to a more productive cytotoxic anti-tumor immune response, consistent with human and murine studies[9,11,12]. Most importantly, we found that following RT combined with pembrolizumab, irrespective of the starting immune composition, nearly 80% of the patients examined demonstrated new and activated CD8 T cell clones compared to less than 20% with pembrolizumab alone, which mirrors previous clinical data for pembrolizumab (Figure 4f and g)[13-15]. These results suggest that RT sensitizes breast tumors to checkpoint blockade by producing new T cell clones and encouraging their activation through the stimulation of key anti-tumor pathways including type I interferons, which have been previously identified as essential mediators of RT-induced inflammation[21,22].

Our most intriguing finding was the discovery of a specific macrophage phenotype that occurs following RT. Previous studies have suggested that eliminating macrophages enhances the response to RT in breast, glioblastoma and head and neck cancers[16-18]. This current study suggests these successes resulted from targeting the immunosuppressive activity of macrophages triggered in the later phases following RT. However, our study revealed macrophages that enter tumors immediately following RT are largely inflammatory and primed to kill damaged cells through their expression of complement. Over the course of a week though, these cells gradually acquire increasing immunosuppressive capacity as they restore tumor hemostasis. During this process macrophages strongly upregulate their digestive pathways and express multiple proteins suggesting elevated phagocytosis and antigen presentation. We hypothesize the synergy between RT and anti-PD-1 therapy lies in the ability to retain killing and antigen presenting capacity while preventing suppressive activity. This hyper-phagocytic phenotype likely transitions to an immunosuppressive one eventually, as macrophages eating apoptotic bodies exhibit increased immunosuppressive capacity[7,19]. Our findings, further highlight the idea that phagocytic checkpoints are critical in regulating tumor responses in part through their ability to regulate macrophage suppressive capacity[20,21].

Thus, in our early project studies we have characterized the entire immune landscape of breast tumors following RT and shown that there are dramatic changes in the numbers, but more importantly, the functional phenotype of the intratumor immune cells. In both preclinical models and patients, we find that targeting the suppressive activity induced by RT using anti-PD-1 therapy leads to significant synergistic increases in cytotoxic activity beyond RT or anti-PD-1 alone. These findings suggest that RT combined with PD-1/PD-L1 directed therapy converts unresponsive breast tumors to responsive ones in the neoadjuvant setting.

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

Nothing to report

How were the results disseminated to communities of interest?

In addition to the project meetings relevant members of the Knott and Shiao labs attend, we also hold general joint laboratory meetings where we discuss the multiple shared projects between our two labs. We have invited and our consumer advocate Michele Rakoff has joined these monthly meetings up until they were canceled due to Covid-19. As we begin these meetings again Michele will be invited to join and to begin to disseminate our results to the advocate and patient community.

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

Our mouse projects have now resumed so a major focus going forward will be pushing to complete the multi-dose experiments, as well as the two tumor experiments to study abscopal responses. We will also begin to look into other cell populations in the microenvironment such as B cells. Further, we will begin to study ligands expressed by specific cell groups and how these may be regulating phenotypes or abundances of other populations. Further, we will also begin to incorporate data from the CD45- fractions of these tumors to understand how the tumor cells, fibroblasts and endothelial cells are changed by the combined therapy and how these cells may be influencing other populations.

In terms of clinical studies, we will continue to accrue patients into our phase II trial. We expect that the number of non-responding patients will begin to reach levels where valid comparisons can be made between responders and non-responders in terms of their immune composition. Further, like with the murine data we will begin to delve deeper into the B-cells and other immune populations in these tumors, and we will begin to analyze the CD45- fractions. Also, like with the mouse experiments, we will begin to study ligand and receptor interactions between different immune and stromal populations so that the cellular interactions governing response can be uncovered.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

Our single cell analysis of the murine and human tumor microenvironment and in particular the macrophage population has allowed us to better describe the transcriptional machinery shaping the phenotypic heterogeneity of each immune cell population. This is particularly true for macrophages, whose categorization into pro-tumor M1 or anti-tumor M2 groups previously has been controversial and generally regarded as not capturing their full phenotypic plasticity. Our analysis of murine and human macrophages has identified four module transcriptional regulatory networks that regulate these populations with similar modules being contained in each network, such as ones regulating inflammation and suppression, phagocytosis, and antigen presentation. We found that radiation strongly induces expression of the phagocytosis and antigen presentation modules and that the addition of anti-PD1 therapy increases expression of these modules. We hypothesize that antigen presentation by these cells after RT is a reason for why there is an increased number of novel CD4+ and CD8+ T cell clones in the tumor.

In terms of clinical practice, our mouse studies and our early clinical studies indicate that the combination of anti-PD1 therapy and RT in the neoadjuvant setting for women with TNBC results in significantly increased rates of pathological complete or near complete response compared to historical value for chemotherapy alone[22]. If this trend continues in the remaining patients it would

indicate the combination is a valid alternative to the combination of anti-PD1 therapy and chemotherapy that was recently reported to increase response rates to 64%[23]. The phase Ib portion also revealed that the proposed strategy is also safe with no additional reported surgical complications (wound healing, infection) and one Grade 3 skin reaction and one Grade 4 incidence of colitis. Thus, from the initial portion of this trial, we determined that our strategy as it stands is safe and may have potential efficacy so we determined with the Institutional Review Board and Data Safety Monitoring Committee to proceed with the phase II portion of the trial which is currently underway.

What was the impact on other disciplines?

Our description of tumoral macrophages and how they regulate immunotherapy response is likely also valid for other tumors such as lung tumors where novel new CD8+ T cell clones are also observed after anti-PD1 therapy with RT, like we see here. More broadly, the transcriptional modules that we have identified as being important for the plastic nature of macrophages are also likely valid for macrophages in other tumors, and perhaps diseases, and these modules could be an area of further studies in those tumors and diseases.

This is a novel strategy incorporating RT upfront in the neoadjuvant setting for triple-negative breast cancer. Thus far, we have determined that it was safe and now we are studying whether it is efficacious compared to the standard of care. This paradigm will change the way RT is incorporated into breast cancer and also have implications for multiple cancers and how they incorporate RT and immunotherapy.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

We experienced a several month delay in our murine experiments and a slowed clinical accrual as a result of COVID19. Due to the laboratory and clinical trial operation shut down we did not complete everything we had hoped to over the past few months. Things have since resumed near normal operations and thus we do not anticipate any further delays.

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Poster Presentation at San Antonio Breast Cancer Conference (2019):
H. McArthur *et al.*, Abstract P2-09-07: Preoperative pembrolizumab (Pembro) with radiation therapy (RT) in patients with operable triple-negative breast cancer (TNBC). *Cancer Research* **79**, P2-09-07-P02-09-07 (2019).

- **Website(s) or other Internet site(s)**

  Nothing to Report

- **Technologies or techniques**

  Nothing to Report

- **Inventions, patent applications, and/or licenses**

  Nothing to Report

- **Other Products**

  Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Simon Knott
Project Role:	Partnering Principle Investigator

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Knott supervised the development and analysis of single cell libraries from mouse tumor models and human tumor biopsies.
Funding Support:	Department of Defense

Name:	Catherine Oh
Project Role:	Research Tech
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Catherine Oh has established pipelines for single cell library preparation from mouse tumors and human tumor biopsies and she will apply these skills to process the mouse and human experimental samples.
Funding Support:	Stand Up to Cancer

Name:	Kenneth Gouin
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Kenneth helped generate libraries and analyze and interpret the corresponding data
Funding Support:	CSMC Institutional Commitment

Name:	Nathan Ing

Project Role:	Research Bioinformatician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Nathan Ing has developed and is developing computational pipelines for processing single cell data from mouse tumor models and human tumor biopsies.
Funding Support:	Department of Defense

Name:	Bassem Ben Cheikh
Project Role:	Research Bioinformatician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Bassem Ben Cheikh has developed and is developing computational pipelines for processing single cell data from mouse tumor models and human tumor biopsies.
Funding Support:	Department of Defense

Name:	Stephen Shiao
Project Role:	Partnering Principle Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.4
Contribution to Project:	Dr. Shiao wrote the IACUC and clinical trial protocols and supervised the training of the personnel who will be conducting the mouse experiments, working on the clinical trial and processing the samples resulting from the clinical trial.

Funding Support:	Department of Defense, National Cancer Institute

Name:	Fleury Nsole-Biteghe
Project Role:	Postdoctoral Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Dr. Nsole-Biteghe helped write the IACUC protocol and work on the experimental protocols needed for the murine experiments.
Funding Support:	Department of Defense

Name:	Regina Henson
Project Role:	Research Assistant I
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Regina Henson has been working to develop the clinical trial protocols for processing clinical specimens and has helped Dr. Shiao with the writing of the lab manual.
Funding Support:	Department of Defense

Name:	Natalie-Ya Mevises
Project Role:	Research Assistant I
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6

Contribution to Project:	Natalie-Ya Mevises has been assisting with development and refinement of the clinical and lab specimen processing.
Funding Support:	Department of Defense

Name:	Heather McArthur
Project Role:	Partnering Principle Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5
Contribution to Project:	Dr. McArthur has been working on the development of the clinical trial protocol.
Funding Support:	Department of Defense

Name:	Scott Karlan
Project Role:	Partnering Principle Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.5
Contribution to Project:	Dr. Karlan has been assisting with the development of the clinical trial protocol, particularly the specifics of the specimen collection.
Funding Support:	Department of Defense

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. REFERENCES

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10. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*