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CONTRACTING ORGANIZATION: Washington University in St. Louis - School of Medicine

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14. ABSTRACT

Triple negative breast cancer (TNBC) remains a challenge to clinicians, laboratory investigators, and patients due to its disproportionate number of breast cancer deaths and its lack of an established therapeutic target. Numerous studies have identified potential novel mutational gene targets in TNBC, but single-agent therapeutics have lacked substantial impact in TNBC. More recently, immune checkpoint inhibitors gained significant clinical traction in breast cancer. Unfortunately, initial promising results have been subsequently overshadowed with failures, particularly in TNBC. In other solid human tumors, the efficacy of anti-PD-L1 immune checkpoint therapies appeared to be enhanced by stimulating lymphocyte infiltration into the tumor microenvironment with type I IFNs. Here in our second year, we show induction of RIG-I and the interferon response can cause an increase in tumor infiltrating lymphocytes in animal models of TNBC.

15. SUBJECT TERMS
ADAR, interferon signaling, RIG-I, triple negative breast cancer

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1. INTRODUCTION

Triple negative breast cancer (TNBC) remains a challenge to clinicians, laboratory investigators, and patients due to its disproportionate number of breast cancer deaths and its lack of an established therapeutic target. Numerous studies have identified potential novel mutational gene targets in TNBC, but single-agent therapeutics have lacked substantial impact in TNBC. More recently, immune checkpoint inhibitors gained significant clinical traction in breast cancer. Unfortunately, initial promising results have been subsequently overshadowed with failures, particularly in TNBC. In other solid human tumors, the efficacy of anti-PD-L1 immune checkpoint therapies appeared to be enhanced by stimulating lymphocyte infiltration into the tumor microenvironment with type I IFNs. We have now identified a similar IFN-stimulated pathway in TNBC. We show that TNBC cells express high levels of the RIG-I double-stranded RNA sensor and downstream active JAK1/STAT1/INF-β pathway components. Moreover, we show that TNBC cells display an interferon gene signature, suggesting that TNBC cells are primed to respond to type I IFNs. Further stimulation of this pathway would result in enhanced expression of PD-L1, a known transcriptional target of IFN-β, as well as the recruitment of IFN-responsive tumor infiltrating lymphocytes. We now propose to build on these exciting preliminary findings generated from our previous award with a series experiments aimed at determining the clinical utility of hyper activating RIG-I and increasing IFN-β production to sensitize TNBC cells to immune checkpoint therapies.

2. KEYWORDS

ADAR, interferon signaling, RIG-I, triple negative breast cancer

3. ACCOMPLISHMENTS

Major Goals of the Project

There was one major milestone for this first year of the grant proposal: determine the expression levels of IFN-β and ISG signature in each cell line and whether cells are sensitive to RIG-I activation or inhibition.

Goals Accomplished

Major Task 4: Determine whether RIG-I and IFN-beta production increase tumor infiltrating lymphocytes in TNBC.

We hypothesized that increased RIG-I activity and subsequent stimulation of IFN-beta production would result in significant gains in tumor infiltrating lymphocytes (TILs) into the tumor microenvironment of TNBC tumors. To test this hypothesis, we utilized two separate in vivo breast cancer models to measure the effects of RIG-I activation. One model uses a well-established 4T1 mouse tumor cell line that we have shown expressed elevated RIG-I and ISG15. Moreover, ISG15 expression in 4T1 cells is significantly lowered upon RIG-I shRNA treatment. The second model utilizes *p53/ARF*-double-null mouse mammary epithelial cells (MMECs) isolated from pure C57Bl6 females that we generated. These cells also exhibit high RIG-I expression and a strong innate immune gene signature. Ten mice from each model were used as internal controls, being injected with parental 4T1 cells (into Balb/c mice) or *p53/ARF*-null MMECs treated with control RNA. An additional ten animals from each model were injected with cells that had been pre-treated with 3pRNA. We housed treated mice for one week following injection of cells into the mammary fat pad. Then, we harvested mammary fat pads containing tumors, paraffin embedded and fixed tissues, and performed immunohistochemical analysis using antibodies recognizing CD3, CD4, and CD8 on separate but consecutive fat pad slices. As shown in **Figure 1A**, CD8+ TILs were quantified in each mouse model, clearly showing an increase in TILs upon 3pRNA treatment.

Major Task 6: Determine whether PDX or immune competent tumors respond to enhanced IFN-beta production.

We also determined whether IFN-beta is sufficient to drive T cell infiltration into the tumor microenvironment. Using the same cells from above, we incubated cells with vehicle control or 500U of purified IFN-beta prior to injection into mammary fat pads of recipient female mice from each background. Following the same one-week time frame, we harvested tissues and processed as described above to measure tumor infiltrating T cells. However, IFN-beta exposure did not directly influence TIL numbers (**Figure 1B**).

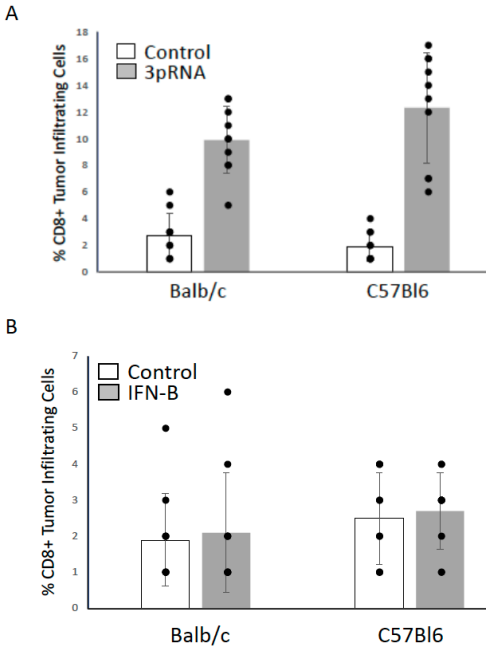

Figure 1. Tumor infiltrating lymphocytes in mouse models of IFN stimulation. A) 4T1 cells or p53/ARF double-null cells were injected into mammary fat pads of Balb/c and C57Bl6 mice, respectively and treated with control or 3pRNA. B) 4T1 cells or p53/ARF double-null cells were injected into mammary fat pads of Balb/c and C57Bl6 mice, respectively and treated with control or IFN-beta. Tumor infiltrating lymphocytes were measured using antibodies recognizing CD8 for all mouse tumors.

Major Task 5: Determine if the ISG signature in TNBC correlates with immune infiltration.

We now provide preliminary data that TNBCs from patients exhibit higher baseline expression of RIG-I, making them potentially receptive to RIG-I activation. However, it is unclear from these human data whether there are sufficient IFNs in the tumor space in the breast to allow for tumor infiltrating lymphocytes. Rather, tumor cells might be primed and ready to produce IFN-beta upon further stimulation of RIG-I, moving over some threshold of production that would entice immune infiltration. The goal of this major task was to establish this possibility in human samples. We have now stained 525 TNBC human patient samples for CD4, CD8, ISG15, and RIG-I (Figure 2). ISG15 expression was elevated in 78% of samples, RIG-I was elevated in 55%, CD4+ samples represented 22%, and CD8+ represented 51% of samples. Overall, this suggests that the pathway is highly elevated in TNBC and that most of these patients also exhibit high infiltration of CD8+ cells, but not CD4+ cells. We are currently conducting a deeper statistical analysis of these correlations to be included in year 3.

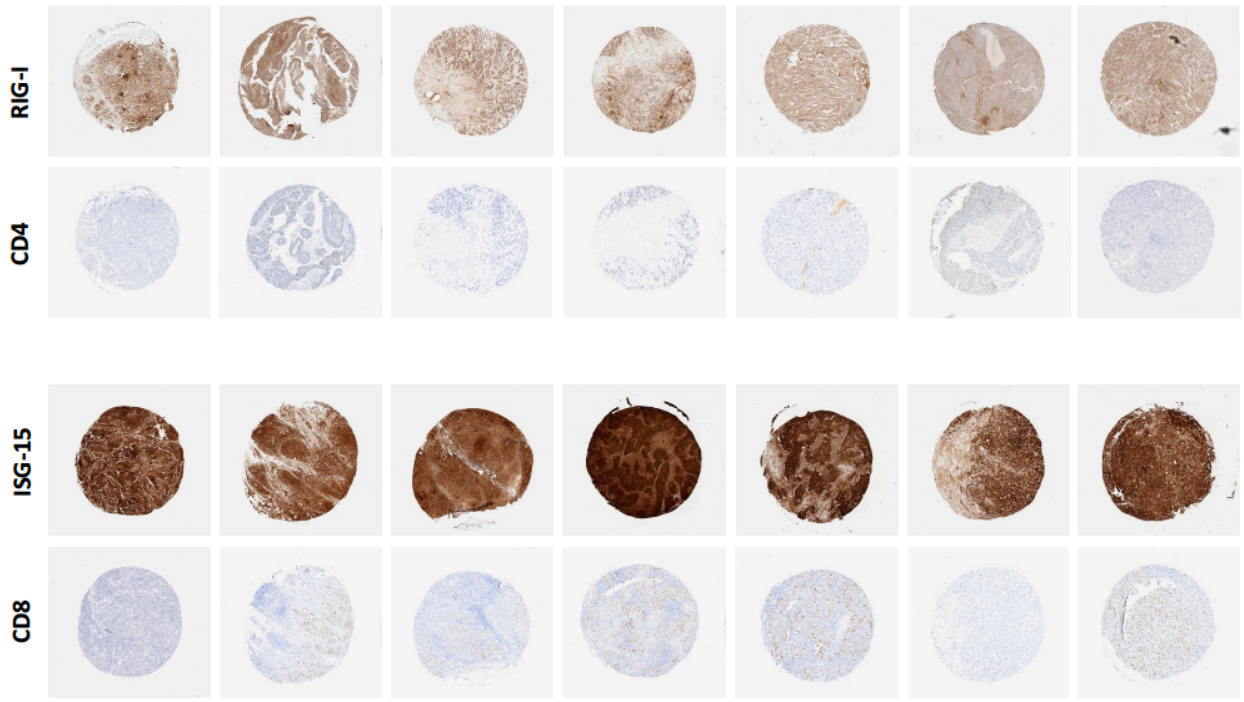

Figure 2. Immunohistochemical staining of 525 TNBC patient samples. A tissue array was stained for each of the indicated antibodies (RIG-I, CD4, ISG-15, and CD8) using a Bond autostainer. Representative images of 7 different TNBC samples for each stain are provided.

Training Opportunities
Nothing to Report

Results Disseminated to the Community
I participated this past year in disseminating our initial findings to three independent groups of large donors to the American Cancer Society. These donors visited my laboratory at Washington University where I discussed the research in this grant proposal and how our results were moving the field of breast cancer research forward. We engaged in a question-and-answer session where the donors queried me on the clinical impact of this work. I anticipate doing this laboratory tour again next year and have already been asked by the American Cancer Society to do so.

Plans for Next Reporting Period
In year 3, we will focus on completing MAJOR TASKS 6 and 7. Specifically, we will finish experiments aimed at determining whether activation of RIG-I results in increased T cell infiltration into implanted tumors. This will also be done following direct IFN-beta stimulation. We have already begun to grow PDX samples in recipient mice. Finally, we will begin treating immune competent mice with IFN-beta, RIG-I agonists and PD-L1 inhibitor.

4. IMPACT
Impact on Principal Discipline
Our current work will be incredibly impactful for those studying TNBC. We have discovered that activation of RIG-I can lead to an increase in tumor infiltrating lymphocytes.

Impact on Other Disciplines
Nothing to Report

Impact on Technology Transfer
Nothing to Report

Impact on Society
We have disseminated the data and ideals from this grant proposal to several groups in the St. Louis community including the American Cancer Society as well as to a general audience at the AACR Disparities in Cancer meeting. They were encouraged by our progress and excited about the future clinical impact our work might provide.

5. CHANGES/PROBLEMS
Changes in Approach
Nothing to Report

Anticipated Problems or Delays
Nothing to Report

Changes in Human, Animal Biohazards and/or Selective Agents
Nothing to Report

6. PRODUCTS
Publications, Conference Papers and Presentations
Nothing to Report

Internet Sites
Nothing to Report

Technologies or Techniques
Nothing to Report

Inventions, Patents and/or Licenses
Nothing to Report

7. PARTICIPANTS
Individuals That Have Worked on Project

Name:	Jason D. Weber
Project Role:	PI
Nearest person month worked:	1.2
Contribution to Project:	Dr. Weber served as the mentor for Drs. Maggi and Cottrell in planning all experiments and overseeing the final data analysis.
Funding Support:	NIH CA262804; W81XWH-21-1-0476; W81XWH-21-1-0391

Name:	Leonard B. Maggi
Project Role:	Co-Investigator
Nearest person month worked:	1.2

Contribution to Project:	Dr. Maggi worked with Dr. Cottrell on all the experiments detailed in year 1.

Name:	Shunqiang Li
Project Role:	Co-Investigator
Nearest person month worked:	0.6
Contribution to Project:	Dr. Li provided expertise on all mouse and patient sample experiments.

Name:	Cynthia Ma
Project Role:	Co-Investigator
Nearest person month worked:	0.3
Contribution to Project:	Dr. Ma provided oncology guidance on all experiments.

Name:	Kyle Cottrell
Project Role:	Postdoctoral Research Associate
Nearest person month worked:	6
Contribution to Project:	Dr. Cottrell worked with Dr. Maggi on all the experiments detailed in year 1.

Changes in Active Other Support for PD/PI
Nothing to Report

Other Organizations Involved as Partners
Nothing to Report