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TITLE: Targeting Neutrophil Protease-Mediated Degradation of Tsp-1 to Induce Metastatic Dormancy

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14. ABSTRACT

Background.
External pre-existing inflammation in the lungs is linked to increased incidence of metastasis. Inflammation –mediated by bacterial infection or cigarette smoke enhanced pulmonary metastasis from breast cancer in humans and mice. Similarly, autoimmune arthritis, characterized by increased recruitment of inflammatory neutrophils and macrophages in the lungs was associated with increased breast cancer metastasis to the lungs. Despite this compelling link between inflammation and metastasis, the mechanisms by which inflammation contributes to tumor outgrowth in distant metastatic organs have remained underexplored. We believe that targeting inflammation-mediated metastasis has tremendous potential in the treatment of high-risk breast cancer patients.
Overarching challenges. Breast cancer affects more than 1.7 million individuals a year worldwide, with approximately 500,000 deaths. Importantly, >90% of this mortality is a consequence of metastatic disease that is resistant to adjuvant therapies. Despite this clinical significance, there is a conspicuous lack of a single FDA approved molecularly targeted anti-metastatic therapy. Hence, there is an urgent medical need to develop new targeted anti-metastatic therapeutic approaches. However, a lack of mechanistic understanding by which tumor cell colonize and outgrow in distant metastatic organs, has been a major impediment to the development of an effective anti-metastatic therapy.
Hypothesis /Objective. We hypothesize that intervention against inflammation-driven neutrophil elastase (NE)/Cathepsin G (CG)-Thrombospondin-1 (Tsp-1) axis can be developed into an anti-metastatic therapy in breast cancer. Our objectives are: 1) to establish that the neutrophil NE/CG-Tsp-1 axis is the dominant pathway in inflammation-mediated metastasis, 2) to determine the molecular mechanisms by which neutrophil CG/NE-Tsp-1 axis promotes metastasis, 3) to show that NE/CG-Tsp-1 axis modulates Tsp-1-mediated metastatic dormancy, 4) to assess whether pharmacological inhibition of CG/NE can be used to inhibit metastasis, and 5) to determine if induction of Tsp-1 expression in the lung microenvironment with a novel DWLPK peptide constitutes an anti-metastatic approach. Our overall goal is to develop a mechanism-guided intervention against inflammation-driven breast cancer metastasis.
Specific Aims. 1) To determine the role of neutrophil NE/CG-Tsp-1 axis in breast cancer metastasis to the lung; 2) To determine if pharmacological inhibition of NE and CG can be used to inhibit metastasis, and 3) To determine if ectopic induction of Tsp-1 expression in the lung microenvironment blocks NE/CG-mediated metastasis.
Study Design. We have recently demonstrated that external inflammation in the lungs is associated with increased incidence of metastasis. We discovered a novel mechanism, whereby abundant neutrophils recruited in the inflamed lungs degranulate their azurophilic granules to release two key serine proteases, CG and NE. These proteases specifically target the tumor suppressor Tsp-1, for proteolysis, to generate tumor-promoting microenvironments. Using a combination of genetic and pharmacological approaches, we will determine the mechanistic role and therapeutic potential of CG/NE-Tsp-1 axis in inflammation-mediated breast cancer metastasis.
Innovation. This proposal addresses the critical and unique link between pre-existing inflammation in the lungs and increased incidence of metastasis from breast cancer. A variety of mouse genetic models, together with compartment-specific gene knockout strategies will be employed. In parallel, pharmacological approaches will be used to complement the genetic strategies, and to provide feasibility for clinical translation. This study emphasizes that therapy should be targeted against the reprogrammed host microenvironment, which contributes to, and supports, the e growth and survival of disseminated tumor cells
Impact. We expect to unravel mechanistic and therapeutic insights and generate unique translational opportunities and may lead to the design of future clinical trials for high-risk breast cancer patients that exhibit inflammation (Cigarette smoke, COPD/emphysema related). Notably, the dual NE/CG protease inhibitor Sivelestat is available and is currently being used in Phase III clinical trials of acute lung injury with systemic inflammatory response syndrome. We expect that findings from our studies will support the potential for repurposing Sivelestat as a dual protease antagonist in the treatment of metastasis in breast cancer patients with lung inflammation. Similarly, induction of Tsp-1 expression with a novel DWLPK peptide drug either alone or in combination with Sivelestat has tremendous potential for designing future clinical trials for high-risk breast cancer patients.

15. SUBJECT TERMS
Triple negative breast cancer, metastasis, lipopolysaccharide, thrombospondin 1, cathepsin G, bone marrow transplantation, neutrophil elastase, sivelestat

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1. INTRODUCTION:

We hypothesize that intervention against inflammation-driven NE/CG- Tsp-1 axis can be developed into an anti-metastatic therapy in breast cancer. Using a combination of genetic and pharmacological approaches, we propose to achieve the following objectives; 1) to establish that the neutrophil NE/CG-Tsp-1 axis is the dominant pathway in inflammation-mediated metastasis, 2) to determine the molecular mechanisms by which neutrophil CG/NE-Tsp-1 axis promotes metastasis, 3) to show that NE/CG-Tsp-1 axis modulates Tsp-1-mediated metastatic dormancy, 4) to assess whether pharmacological inhibition of CG/NE with Sivelestat can be used to inhibit metastasis, and 5) to determine if induction of Tsp-1 expression in the lung microenvironment with a novel DWLPK peptide constitutes an anti-metastatic approach.

This project addresses BCRP overarching challenges of revolutionizing treatment regimens by replacing interventions that have life-threatening toxicities with ones that are safe and effective; and for advancing the field towards the elimination of mortality associated with metastasis in high-risk breast cancer patients. It also addresses metastatic dormancy, and progression of breast cancer to life threatening metastasis. In summary, we anticipate that the proposed studies will lead to exciting and novel findings that have the potential to impact inflammation-mediated metastasis in breast cancer.

2. KEYWORDS:

breast cancer, metastasis, Thrombospondin 1, neutrophil, inflammation, metastases

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim1: To determine the role of neutrophil NE/CG-Tsp-1 axis in breast cancer metastasis to the lung.

Major Task 1: Determine if the metastasis-suppressive phenotype in NE-/-CG-/- mice can be rescued in Tsp-1-/- mice.

Subtask 1: Generate cohorts of WT, Tsp-1-/- and TKO BMT mice
Subtask 2: Generate LPS-mediated inflammation in WT, Tsp-1-/- and NE-/- CG-/- Tsp-1-/- mice, administer tumor cells (EO771& PyMT).
Subtask 3: Resect primary tumors and evaluate metastasis in lungs. Characterize phenotypes. Animals: (n=15 per cohort × 3 cohorts × 2 tumor models X repeat expt): 180 mice

Major Task 2: Determine whether loss of NE/CG-Tsp-1axis impacts metastasis by regulating angiogenesis, or proliferation/apoptosis of tumor cells via Tsp-1 receptor CD36.

Subtask 1: Generate shRNA-mediated loss of CD36 expression in tumor cells
Subtask 2: Administer WT and shRNA- tumor cells into WT, Tsp-1-/- and NE-/- CG-/- BMT mice. Animals: (n=15 per cohort × 3 cohorts × 2 tumor models X 2shRNA X repeat): 360 mice

Major Task 3: Determine if Tsp-1 in the lung modulates metastatic dormancy in WT, Tsp-1-/- and NE-/- CG-/- BMT mice. (n=15 per cohort × 3 cohorts × 2 tumor models X repeat): 180 mice

Milestone(s) Achieved: Generation of TKO mice, establish role of NE/CG-Tsp-1 axis. CD36 receptor in inflammation-mediated metastasis, Metastatic dormancy

Aim 2: To determine if pharmacological inhibition of NE and CG can be used to inhibit metastasis.

Major Task 1: Pharmacological inhibition of NE/CG with Sivelestat in WT, Tsp-1-/- NE-/-CG-/- and TKO BMT mice. (n=15 per cohort × 4 cohorts × 2 tumor models X repeat): 240 mice

Major Task 2: Efficacy of Tsp-1-mimetic peptide in inhibiting angiogenesis Tsp-1 deficient lungs. ABT-510 peptide in inhibiting angiogenesis. (n=15 per cohort × 4 cohorts × 2 tumor models X repeat): 240 mice

Milestone(s) Achieved: Determine pharmacological efficacy of Sivelestat and ABT-510 in inflammation-mediated metastasis.

Aim 3: To determine if induction of Tsp-1 expression in the lung microenvironment blocks NE/CG-mediated metastasis.

Major Task 1: Evaluate efficacy of DWLPK peptide in WT, Tsp-1-/- and NE-/- CG-/- BMT mice (n=15 cohort × 3 cohorts × 2 tumor models X repeat): 180 mice+ 30 (scramble controls)= 210 mice
Combine DWLPK with Sivelestat in WT LPS challenged cohorts only (n=15 per cohort × 3 cohorts × 2 tumor models X repeat): 360 mice

Milestone(s) Achieved: Demonstrate efficacy of DWLPK and combined DWLPK and sivelestat in metastasis.

What was accomplished under these goals?

For this reporting period, we are reporting progress for the following:

<u>Aim 1: To determine the role of neutrophil NE/CG-Tsp-1 axis in breast cancer metastasis to the lung.</u>

<u>Major Task 2</u>: Determine whether loss of NE/CG-Tsp-1axis impacts metastasis by regulating angiogenesis, or proliferation/apoptosis of tumor cells via Tsp-1 receptor CD36.

<u>Subtask 1:</u> Generate shRNA-mediated loss of CD36 expression in tumor cells

In the last progress report, we had shown increased expression of CD36 in the metastatic tumor cells. We had indicated that we were using CRISPR-Cas9 to generate CD36 knockouts.

Here we report progress in the generation of CD36 knockout. The sequences of CD36 guide RNAs (gRNA) were successfully cloned into plasmids and TNBC cells were transduced. We used two different guides to introduce CD36 deletion in E0771 TNBC cell line (guide #4: 5'-CCAAAACTGTCTGTACACAG-3' and guide #6: 5'-TTAATCATGTCGCAATAGCT-3'). A scambled gRNA was used as a control. Western blot analysis was used to show loss of CD36 protein in cells transduced with each of the guides (g4 and g6) compared to scrambled controls (**Fig. 1**).

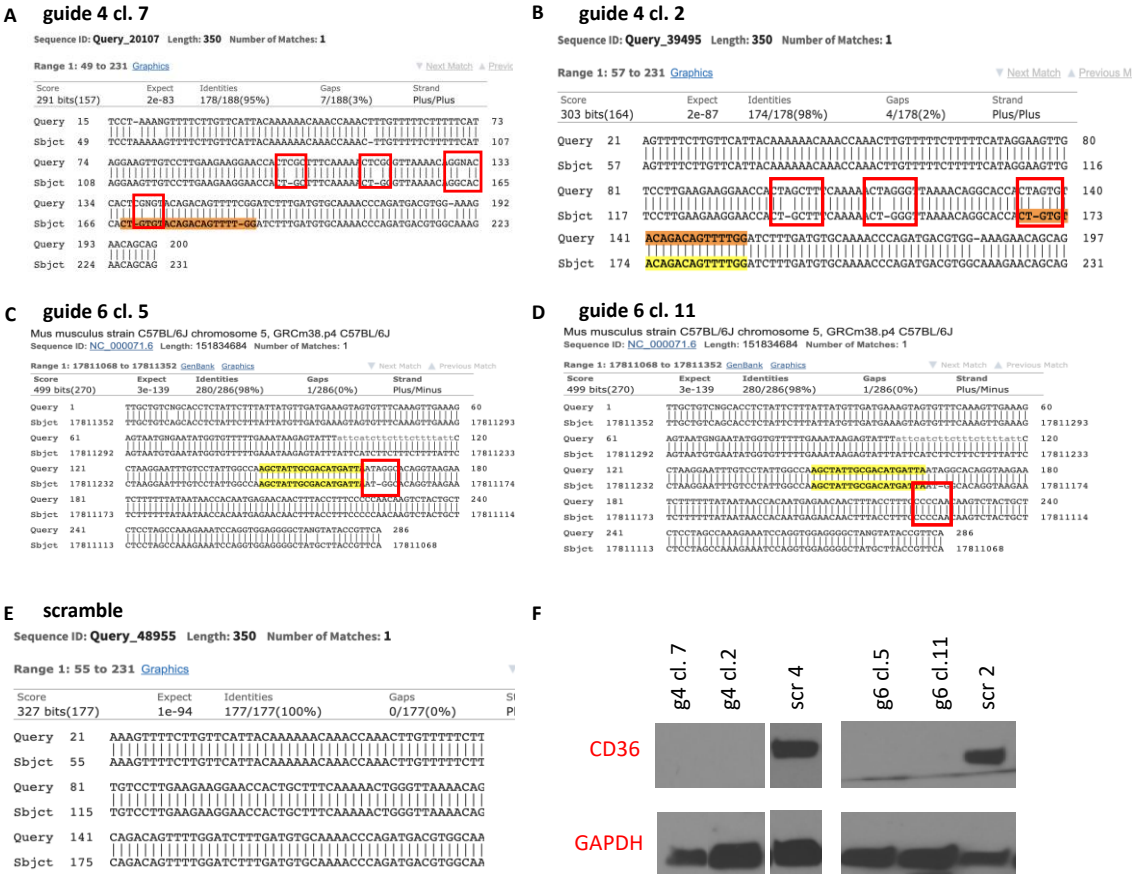

Figure 1. CRISPR-Cas9 induced changes in CD36 DNA with guide #4 (A-B) and guide #6 (C-D). (E) CD36 sequence with scrambled guide. (F) Protein expression of CD36 knockout clones vs. scrambled (scr).

Subtask 2: Administer WT and shRNA- tumor cells into WT, Tsp-1-/- and NE-/- CG-/- BMT mice. Animals: (n=15 per cohort × 3 cohorts × 2 tumor models X 2shRNA X repeat): 360 mice

We analyzed the impact of CD36 loss on cell viability in E0771 cell line. Two independent clones for each of the guides were subjected to cell titer glo assay (**Fig. 2**). Cell growth kinetics of CD36-/- clones wasn't altered significantly when compare to scrambled clones. We will further mix the two independent clones from each guide respectively to generate polyclonal cells for in vivo studies.

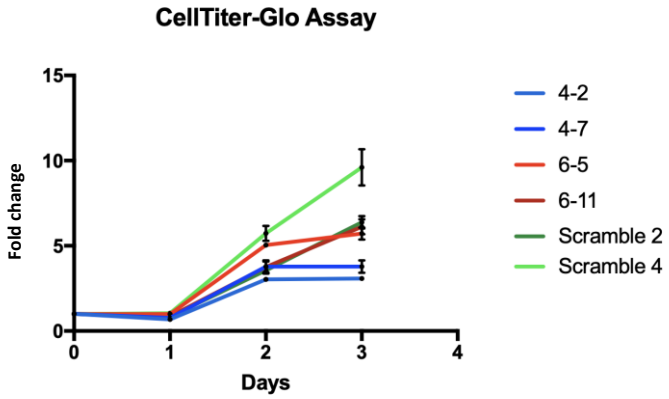

Figure 2. Cell viability of different clones over 72h.

We will combine two clones together for guide #4, #6 and scrambled to develop polyclonal knock-out and scr cells for the in vivo experiment as described below (**Fig. 3**). Previous work from our lab (Catena et al. Cancer Discovery. 2013) demonstrated that a 5-amino acid peptide (Psap peptide DWLPK) has the ability to to induce Tsp-1 by bone marrow Gr1+ cells. Base on this finding, we will utilize the Psap peptide DWLPK to induce the expression of TSP1 *in vivo*. (Figure 3.) Following establishment of metastases we will administer the peptide. We hypothesize that CD36-/- primary tumor cells will lose the ability to activate the TSP-1-CD36 axis at metastatic sites with or without the induction of TSP1 expression. The lack of TSP-1-CD36 axis engagement will further lead to higher tumor burden in mice injected with E0771 CD36-/- clones.

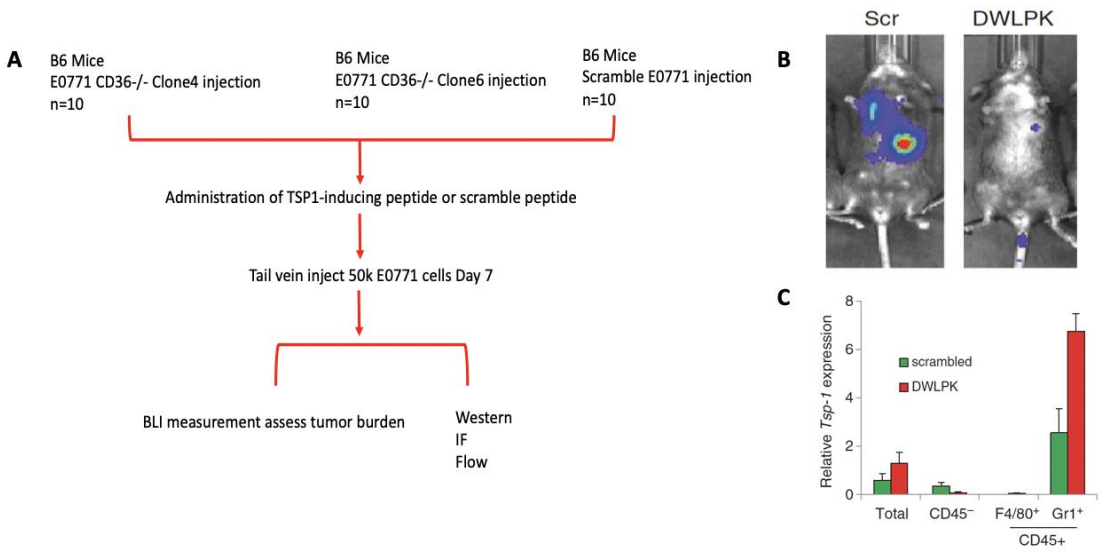

Figure 3. A) Scheme for *in vivo* metastasis experiments, the Psap peptide DWLPK and the scrambled peptide LPKDW will be diluted in saline and administrate to mice (30 mg/kg) via intraperitoneal injection on a daily basis for 6 days before tumor cell injection and until the end of the metastasis assays. Tumor burden and expression of TSP1 and CD36 will be evaluated by BLI and IHC respectively. B) Representative BLI images of animals (n = 5 per group) showing suppression of lung metastases following tail vein injection of tumor cells in mice treated with DWLPK as compared with a scrambled peptide (Scr) as described before. C) Quantitative RT-PCR showing Tsp-1 levels in total lungs and flow cytometry–sorted CD45 − cells and CD45 + cells (F4/80 + macrophages and Gr1 +myeloid cells) from metastases-bearing mice treated with DWLPK compared with scrambled peptide (scrambled; n = 3 per group) as described previously.

What opportunities for training and professional development has the project provided?
Opportunities for training and professional development on the project include the mentorship of post-doctoral associates to help advance their careers.

How were the results disseminated to communities of interest?
In Year 3, Dr. Mittal and the post-doc Dr. Ramchandani have given invited seminars and poster presentations on this topic at the following symposia:

1) NCI PSOC Annual Retreat Ithaca, NY Feb 2019 (poster & seminar)
2) Keystone Symposia, Galveston, TX Feb 2019 (poster)
3) Cold Spring Harbor Labs, Cold Spring Harbor, NY May 2019 (seminar)
4) TME-NYC (Tumor Microenvironment-NYC) Symposium, New York, NY July 2019 (poster & seminar)

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

1) We will combine two clones together for guide #4, #6 and scrambled to develop polyclonal knock-out and scr cells for the in vivo experiment.

2) Utilize the Psap peptide DWLPK to induce the expression of TSP1 *in vivo*.

3) Following establishment of metastases we will administer the peptide.

5) Characterization of the TSP1-CD36 axis in the metastatic phenotype

6) Administer E0771 TNBC tumor cells in Tsp-1-/- and NE-/- CG-/- Tsp-1-/- mice in the context of LPS-mediated inflammation

7) Check the combined efficacy of the peptide and sivelestat in the inhibition of angiogenesis.

8) Check the combined efficacy of the peptide and sivelestat in the inhibition of metastasis.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
In year 1, we have focused on generating reagents and strategies for the planned in vivo studies. In year 2, we began in vivo experiments to interrogate the metastatic cascade as well as metastatic dormancy. In year 3, we were successful in the generation of the CD36 knockout and further characterizing the metastatic phenotype in this model. We will continue to build on these successes.

We anticipate that the proposed studies will lead to novel findings that have the potential to finally impact inflammation-mediated metastasis in breast cancer.

What was the impact on other disciplines?

Progress in elucidating inflammation-mediated metastasis pathways is likely to attract many investigators across disciplines in breast cancer research and result in rapid advancements towards finding a potential therapy against metastatic breast cancer.

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:

The triple-negative E0771 cells that we want to use in our experiments are slightly hard to grow from single clones. It took us time to optimize those conditions and finally obtain cell lines from single clones of the two guides. We recently received the peptides for the in vivo experiment, which took a while to generate.

6. PRODUCTS:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Vivek Mittal (PD/PI) – 15% Effort*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	*Dr. Mittal led the project and oversaw all aspects of the strategy for planning experiments, etc.*
Funding Support:	

Name:	*Divya Ramchandani, PhD (Post-Doc) – 33% Effort*
Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*

Contribution to Project:	*Dr. Ramchandani has performed all neutrophil degranulation assays, flow cytometry, and in vivo experiments*
Funding Support:	

Name:	*Sharrell Lee (Technician) – 50% effort*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Ms. Lee has assisted Dr. Ramchandani on the in vitro work, flow cytometry, animal handling. Most importantly, she has worked on the knockout model. Ms. Lee has helped design and manage the animal experiments.*
Funding Support:	

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

No

What other organizations were involved as partners?

None

8. **SPECIAL REPORTING REQUIREMENTS**
   Nothing to report

9. **APPENDICES:**
   Nothing to report