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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.

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.

Major Task 3: Determine if Tsp-1 in the lung modulates metastatic dormancy in WT, Tsp-1-/- and NE-/- CG-/- BMT mice.

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.

Major Task 2: Efficacy of Tsp-1-mimetic peptide in inhibiting angiogenesis Tsp-1 deficient lungs. ABT-510 peptide in inhibiting angiogenesis.

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. Combine DWLPK with Sivelestat in WT LPS challenged cohorts only.

What was accomplished under these goals?

Aim 1: 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 TKO (CG-/-;NE-/-;Tsp-1-/-)BMT mice

To generate the triple knockout (CG-/-NE-/-Tsp-1-/-) mice we used the breeding strategy shown in Fig. 1A. We had generated TKO heterozygous mice and were expecting to have the homozygous TKO from breeding these mice. However, the average litter size for the F2 generation breeders (**Fig. 1A**) was small, ranging from 4-8 pups. Also, given the low expected frequency of an F2 second generation breeder (1/8) as shown in Fig. 1a, the odds of getting a TKO are even lower. Ultimately, to achieve a better breeding strategy and thus better allele frequency, at best we planned to generate crosses of TKO x TKO (all offspring TKO), or at least TKO X CG-/-NE-/-/Tsp1+/- (1/2 offspring TKO), which would be F3 generation. However, to generate a sufficient cohort size, multiple breeders of these types are required for experiments,

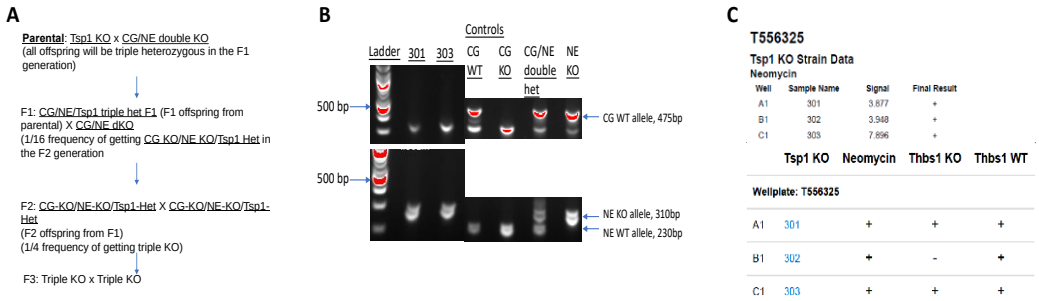

Figure 1. A) Breeding Strategy to generate NE/CG/Tsp-1 triple KO mice. B) Cathepsin G (CG) and Neutrophil Elastase (NE) PCR genotyping. CG WT allele is 475bp, absence of 475bp allele indicates CG KO. NE WT allele is 230bp and NE KO allele is 310bp. C) Transnetyx raw data for Tsp1 (neomycin), and genotyping data (the signal is calculated by normalizing the raw data from Tsp1 probe to a housekeeping gene for Tsp1. 301 (male) and 303 (female) are CG KO/NE KO/Tsp1 Het, which were subsequently crossed to each other to generate a triple KO, frequency 1:4.

which required at least 2-3 litters from the F2 generation. As a result, we reached a standstill at the F2 generation of obtaining TKO for line expansion to yield sufficient animals for experiments. Another bottleneck is that from F2 generation, inspite of getting several litter droppings, we only obtained 2 TKO pups, both of which were males, and thus we were unable to set the F3 breeder (**Fig. 1B-C**). Therefore, we decided to alternatively use Sivelestat to generate NE/CG loss of function in a Tsp-1-/- mice (**Fig. 2**).

Subtask 2. Generate LPS-mediated inflammation in WT, Tsp-1-/- and NE-/- CG-/- Tsp-1-/- mice, administer tumor cells.

The goal was to demonstrate if metastasis-suppressive phenotype in NE-/-CG-/- mice can be rescued in Tsp-1-/- mice. Given that we were unable to generate triple KO mice, we treated Tsp1 KO mice with Sivelestat, an inhibitor of NE and CG to achieve deficiency of NE, CG and Tsp1, as illustrated (**Fig. 2A**).

To generate local lung inflammation, LPS was administered intranasally in a 50 µl volume at a concentration of 0.25 mg/ml every three days in wild-type, Tsp-1 KO, wild-type treated with Sivelestat and Tsp-1 KO treated with Sivelestat. As expected in the absence of Sivelestat treatment, Tsp-1 KO mice showed increased metastasis compared to wild-type mice (**Fig. 2B-C**). Importantly, compared to wild-type mice Sivelestat was unable reduce metastasis in Tsp-1 KO mice (**Fig. 2B, D**), indicative of a functional NE/CG-Tsp1 axis in inflammation-dependent metastasis. However, unexpectedly, metastatic burden in Tsp-1 KO group treated with Sivelestat was lower compared to Tsp-1 KO mice **(Fig. 2B, D)**. These findings suggest that Sivelestat may also have an effect independent of the NE/CG-Tsp1 axis in regulating metastasis.

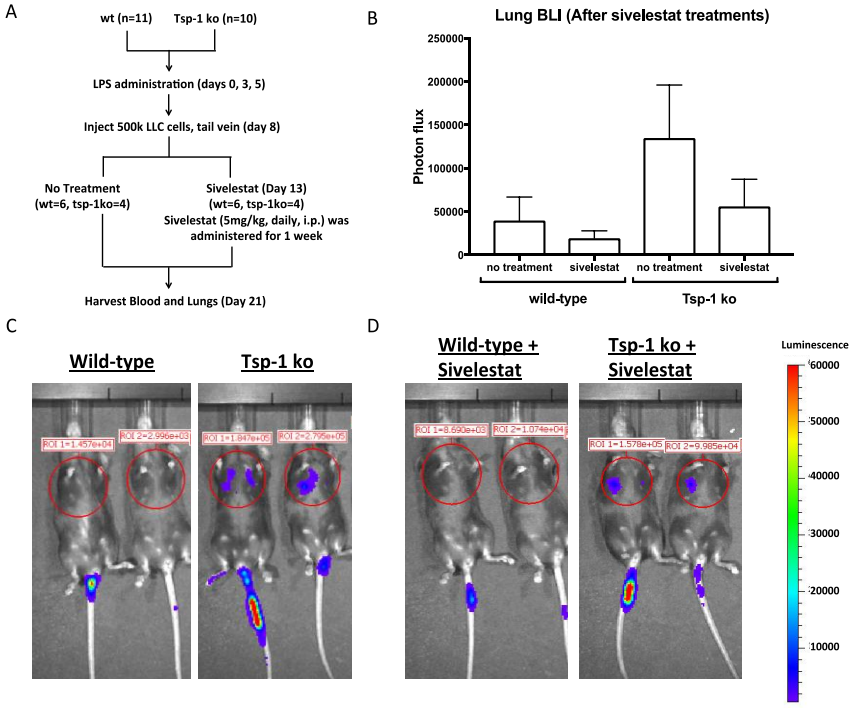

Figure 2. Tsp-1 KO enhances lung metastases. (A) Schematic of experimental design. (B) Lung BLIs after LLC cells were injected via tail vein, but before any Sivelestat treatments. (C) Lung metastasis were higher in tsp-1 ko mice, and inhibition of NE/CG via sivelestat treatments was able to reduce lung tumor burden. (D-E) Representative BLI images of the four cohorts.

Next, we explored the effect of Sivelestat on the tumor microenvironment. First, we performed a comprehensive analysis of bone marrow-derived immune cell subsets in the peripheral blood. Blood samples were collected via retro-orbital sinus puncture into anticoagulant EDTA tubes. PBMCs were extracted using ficoll. Cell pellets were washed with FACS buffer by centrifuging at 1500 RPM for 5 minutes. For surface stains, samples were blocked with anti-mouse CD16/32 for 15 minutes at room temperature, incubated with primary antibodies for 45 minutes in dark on ice, washed with FACS buffer, fixed with 1% formaldehyde for 30 minutes at room temperature in dark, washed with FACS buffer, resuspended in FACS buffer and stored at 4ºC until analysis, which was performed within 24 hours of staining. All antibodies were

obtained from Biolegend and used at a dilution of 1:100. We did not see any differences in any cell type (**Fig. 3-4**), except neutrophils (**Fig. 3A**), which were higher in the blood of Tsp-1 KO mice without Sivelestat treatment.

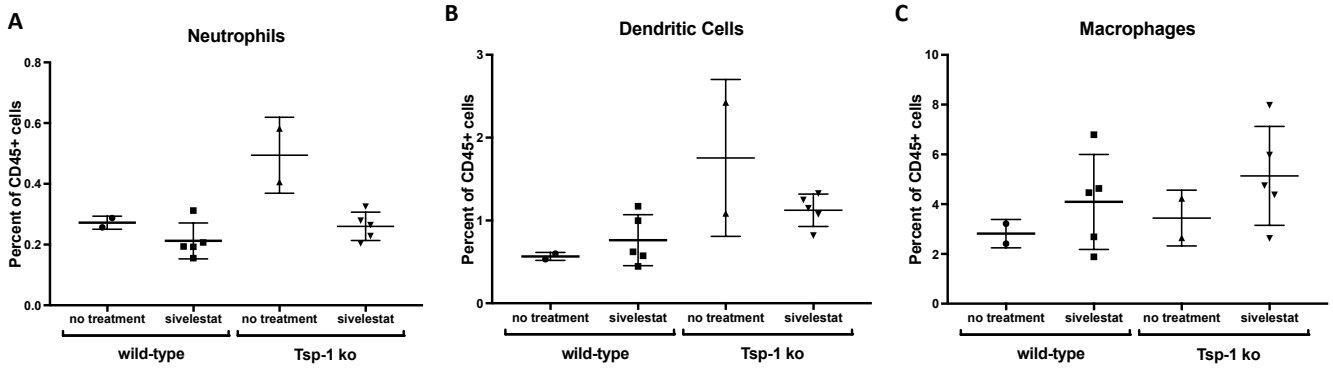

Figure 3. Impact of Sivelestat treatments on myeloid population in blood was analyzed for the four cohorts. No change was observed in the number of dendritic cells (B) or macrophages (C) among the four groups. However, neutrophils (A) seem to be higher in Tsp-1 ko mice over wild-type or Sivelestat-treated groups.

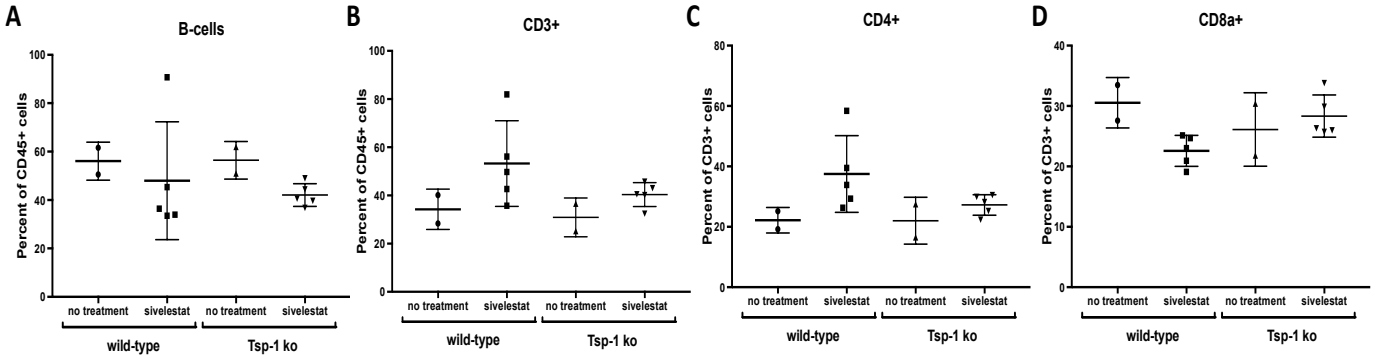

Figure 4. Sivelestat treatment did not impact the total number of B-cells (A), CD3+ (B), CD4+ (C), or CD8+ (D) cells.

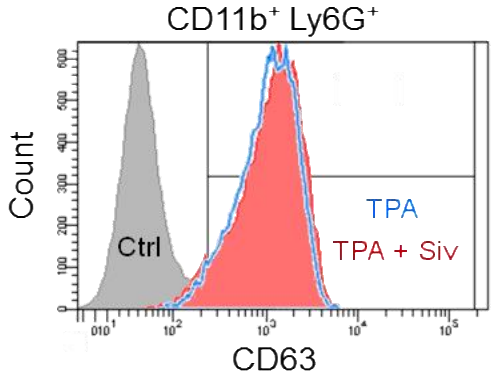

Figure 5. Representative flow cytometry analysis of degranulation marker CD63 in CD11b$^+$Ly6G$^+$ cells cultured *in vitro* with 0.01%DMSO (Ctrl, solid grey histogram), 20nM TPA (TPA, empty blue histogram), or 20nM TPA + 0.05 µg/µl Sivelestat (TPA + Siv, solid red histogram).

Fig 2. Representative flow cytometry analysis of degranulation marker CD63 in CD11b$^+$Ly6G$^+$ cells cultured *in vitro* with 0.01% DMSO (Ctrl, solid grey histogram), 20 nM TPA (TPA, empty blue histogram), or 20 nM TPA + 0.05 µg/µl Sivelestat (TPA + Siv, solid red histogram). Repeated 3 times with similar results.

Given that neutrophil degranulation mediates release of proteases neutrophil elastase (NE) and cathepsin G (CG), we have also established a flow cytometry method to determine degranulation of neutrophils. In this assay, we monitor measuring cell surface presentation of the azurophilic granule membrane molecule, CD63. Increased neutrophil degranulation was associated with enhanced presentation of CD63 (**Fig. 5**). Gr1+ cells are short-lived *in vitro*, hence their viability in culture might be a limiting factor to the study of Tsp-1 induction. To enhance Gr1+ cell viability, we cultured Gr1+ cells in the presence of cytokines like G-CSF or GM-CSF, which enhance viability of Gr1+ cells *in vitro* for several days. Moreover, we also co-cultured isolated Gr1+ cells on a feeder layer of E4ORF1+ HUVECS, which is a system that allows the expansion of hematopoietic stem cells (HSCs) and CD11b+ Gr1+ cells enhanced viability after 2 days in culture (**Fig. 6**). This system provides us an opportunity to study metastatic

pathways in a highly viable *in vitro* model and for the generation of stable cells for any *in vivo* work.

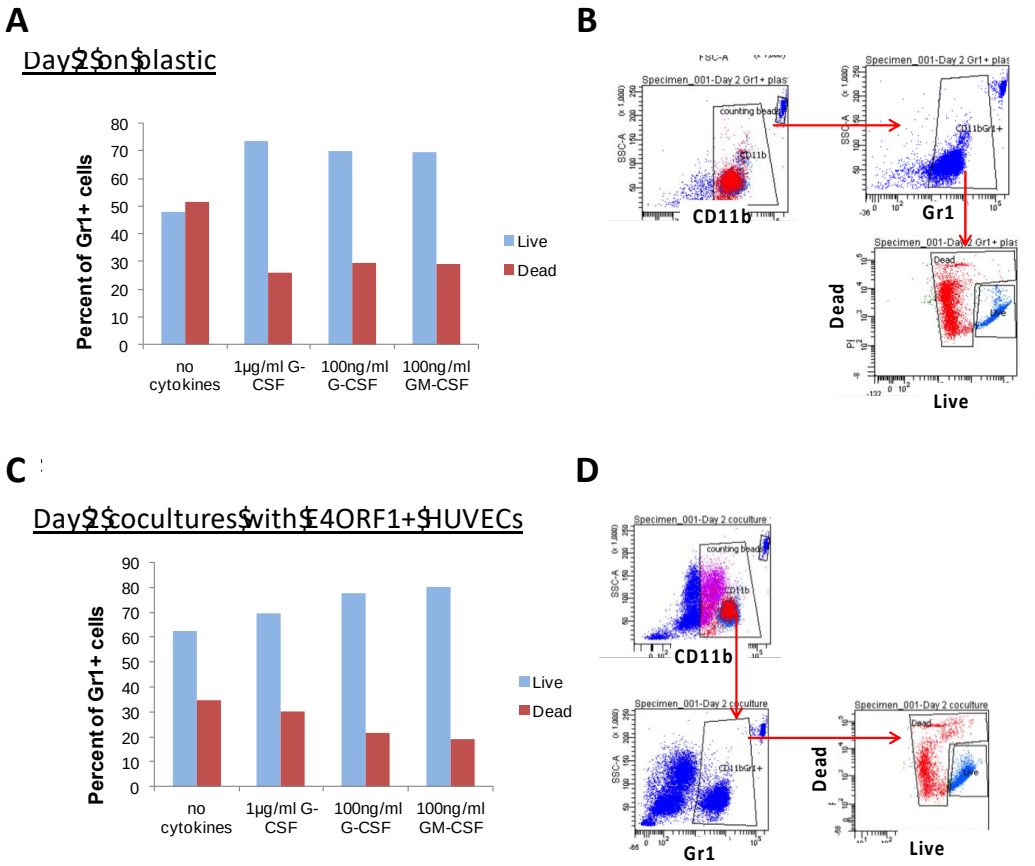

Figure 6. Culturing BM-isolated Gr1+ cells with cytokines on plastic or on a feeder layer of E4ORF1+ HUVECs enhances their viability after 2 days in culture. A) Percentage of live and dead Gr1+ cells after 2 days in cuture on plasticin the absence of added cytokines (no cytokines) or in the presence of 1**µ**g/ml G-CSF, 100ng/ml G-CSF, or 100ng/ml GM-CSF. B) Representative flow cytometric analysis showing the gates used for CD11b+Gr1+ cells, and live vs. dead cells to plot the graph in A. C) Percentage of live and dead Gr1+ cells after 2 days in coculture with E4ORF1+HUVECs in the absence of added cytokines (no cytokines) or in the presence of 1**µ**g/ml G-CSF, 100ng/ml G-CSF, or 100ng/ml GM-CSF. D) Representative flow cytometric analysis showing the gates used for CD11b+Gr1+ cells, and live vs. dead cells to plot the graph in C.

Subtask 3: Resect primary tumors and evaluate metastasis in lungs. Characterize phenotypes.
We used an immunocompetent EO771 primary TNBC model in C5BL/6 mice (Wild-type or Tsp-1 KO, n = 12/group). 100k tumor cells in HBSS were injected into the fourth mammary fat pad of mice. The tumors were allowed to grow until they reached 1 cm^3, at which point they were resected, and mice were monitored for lung BLIs until they died of lung tumor burden to generate a survival curve. We did not see any significant difference in lung metastasis of Tsp-1 KO mice compared to wild-type cohort. Furthermore, the overall

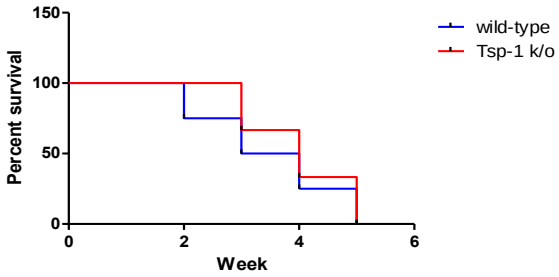

Figure 7. Survival of Tsp-1 KO mice over wild-type group in EO771 primary breast tumor model. X-axis depicts weeks after primary tumor resection.

survival for the two groups remained unchanged (**Fig. 7**). There is a possibility that the primary breast tumors may have systemically reprogrammed the metastatic lungs, which may have neutralized the metastasis promoting effect of Tsp-1 loss.

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.

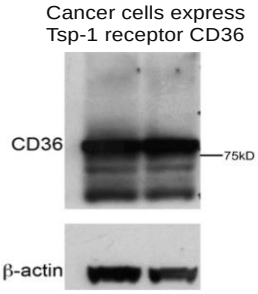

Figure 8. (A) Protein expression of CD36 in tumor cells.

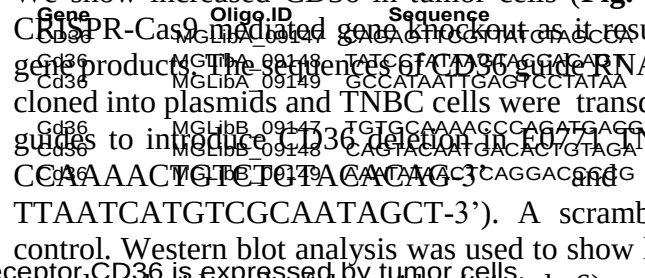

Figure 9. Design of CD36 CRISPR-Cas9 gRNAs for generating biallelic depletion of CD36.

Subtask 1: Generate shRNA-mediated loss of CD36 expression in tumor cells. We show increased CD36 in tumor cells (**Fig. 8**). We have decided to use CRISPR-Cas9-mediated gene knockout as it results in complete depletion of gene products. 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 EO771 TNBC cell line (guide #4: 5'-CCAAAACTGTCTGTACACAG-3' and guide #6: 5'-TTAATCATGTCGCAATAGCT-3'). A scrambled 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. 9**).

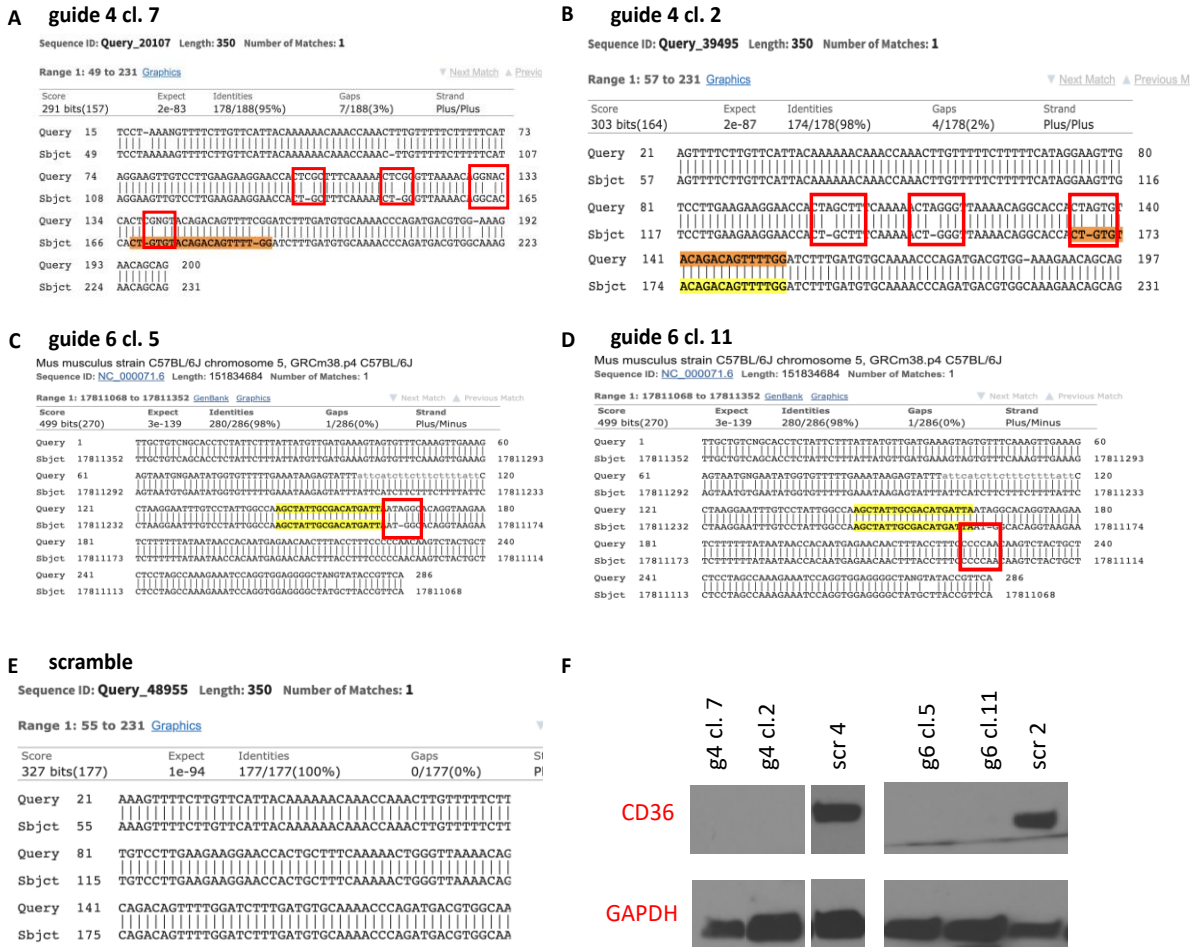

Figure 9. CRISPR-Cas9 induced changes in CD36 DNA with guide #4 (A-B) and guide #6 (C-D). (E) CD36 sequence with scrambled guide. (F) Protein expression of CD36 knockout clones vs. scrambled (scr).

Subtask 2: Administer WT and shRNA- tumor cells into WT, Tsp-1-/- and NE-/- CG-/- BMT mice.

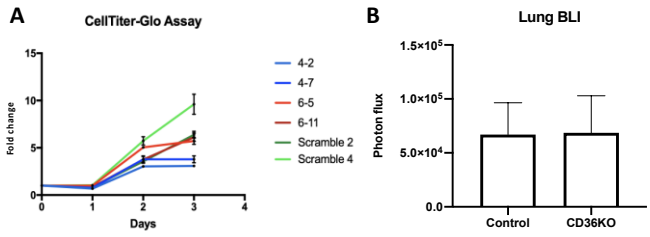

Figure 10. A) Cell viability of different clones over 72h. B) Lung BLI after administration of control and CD36KO clones via tail vein at Day 11.

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. 10A**). Cell growth kinetics of CD36-/- clones wasn't altered significantly when compare to scrambled clones. When we injected control vs. CD36KO EO771 cells in wild-type mice via tail vein, we did not see a significant difference between the growth kinetics of the two groups at day 11 after injection (**Fig. 10B**).

Major Task 3: Determine if Tsp-1 in the lung modulates metastatic dormancy in WT, Tsp-1-/- and NE-/- CG-/- BMT mice.
EO771 TNBC cells expressing mCherry and luciferase reporters were administered in the mammary glands. Tumor growth was monitored and primary tumors were resected. Single disseminated cells in the metastatic lungs (mcherry+) expressed dormancy markers including p38, p21 and low pERK (**Fig. 11**), as low pERK/p38 signaling ratio is associated with induction of both quiescence and survival signaling, which leads to dormancy (Aguirre-Ghiso et al., 2013).

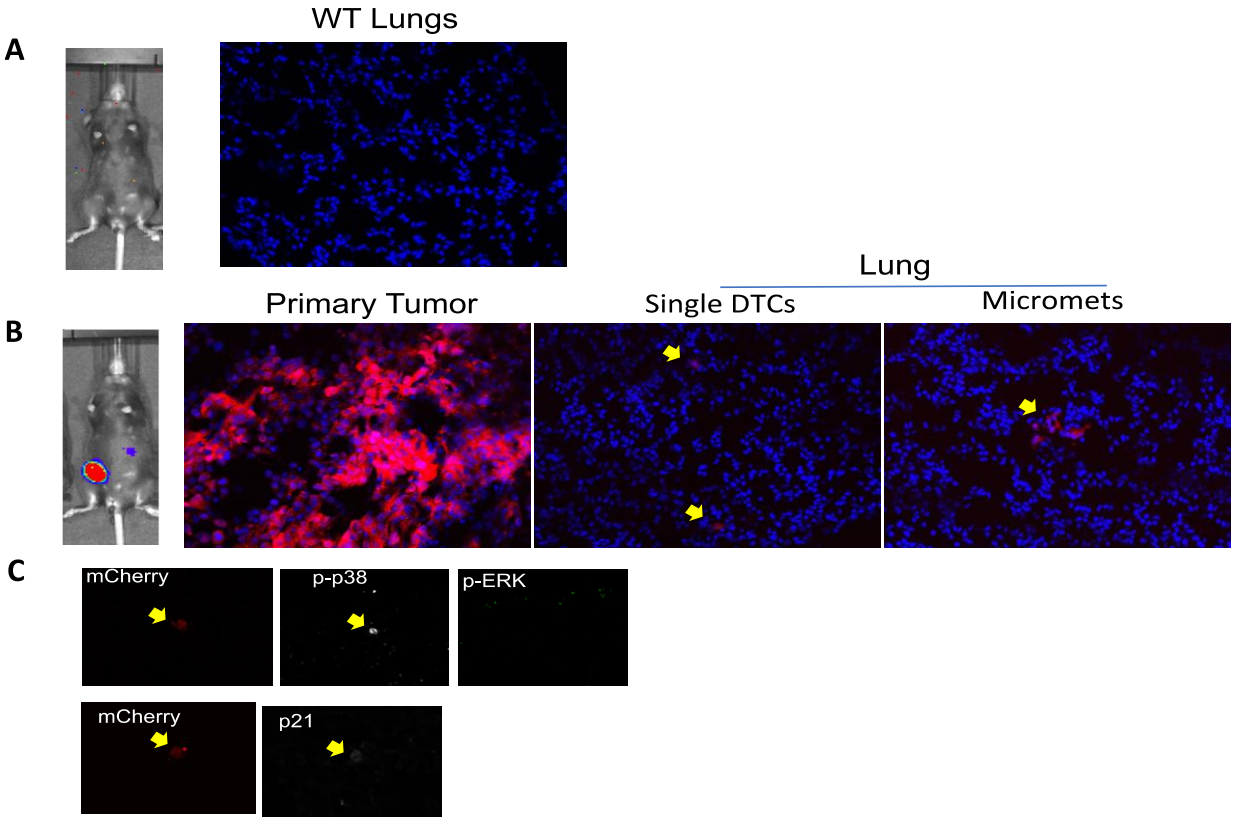

Figure 11. Measurement of metastatic dormancy from EO771 primary tumors. A) Lungs from a wild-type non tumor bearing mice. B) Primary tumors and lungs from mice implanted with mCherry+luc+ EO771 cells in the mammary gland. C) Single disseminated mCherry+ cells in lungs exhibit dormancy phenotypes, including high expression of P-p38, p21 and low expression of P-ERK.

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

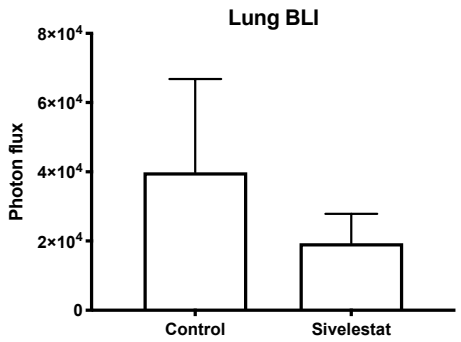

Figure 12. Effect of Sivelestat on tumor-bearing wild-type mice.

Using a tail vein model of 500k LLC tumor cells injected in C57Bl6/j mice after LPS treatment, we found that mice treated with Sivelestat had small lung tumor burden as compared to control mice, without any Sivelestat treatment (**Fig. 12**). The effect of Sivelestat in Tsp-1 KO mice is depicted in **Fig. 2**.

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

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. Based on this finding, we utilize the Psap peptide DWLPK to induce the expression of Tsp-1 *in vivo*. (**Fig. 13**).

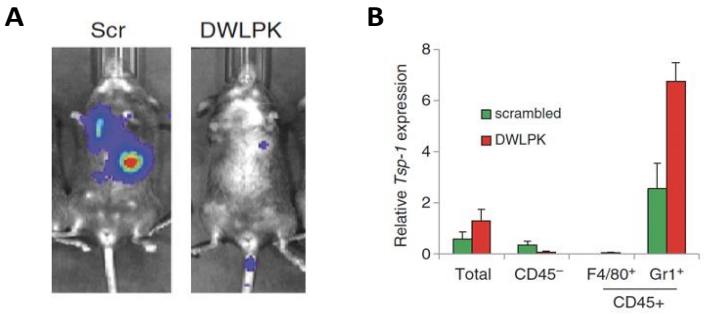

Figure 13. A) Representative BLI images of animals (n = 5 per group) showing suppression of lung metastases following tail vein injection of tumor cells in mice treated with DWLPK as compared with a scrambled peptide (Scr) as described before. B) Quantitative RT-PCR showing Tsp-1 levels in total lungs 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.

To evaluate whether CD36-/- primary tumor cells will lose the ability to activate the Tsp1-CD36 axis at metastatic sites with or without the induction of Tsp1 expression using DWLPK, we will follow the schematic shown in **Fig. 14A**. We observed that administration of DWLPK peptide induced significantly lower tumor in mice injected with control cell line without CD36KO, while CD36KO in EO771 cells led to disabling of Tsp1-CD36 axis and led to a higher tumor burden in

mice (**Fig. 14B**). CD36KO cells without any administration of the peptide were used as controls for optimal tumor growth. Quantification of lung BLI data confirms the regulatory role of Tsp1-CD36 axis in suppressing lung metastatic outgrowth (**Fig. 14C**). Finally we confirmed the increased Tsp-1 expression in lung lysates of mice treated with DWLPK, compared to vehicle treated mice (**Fig. 14D**).

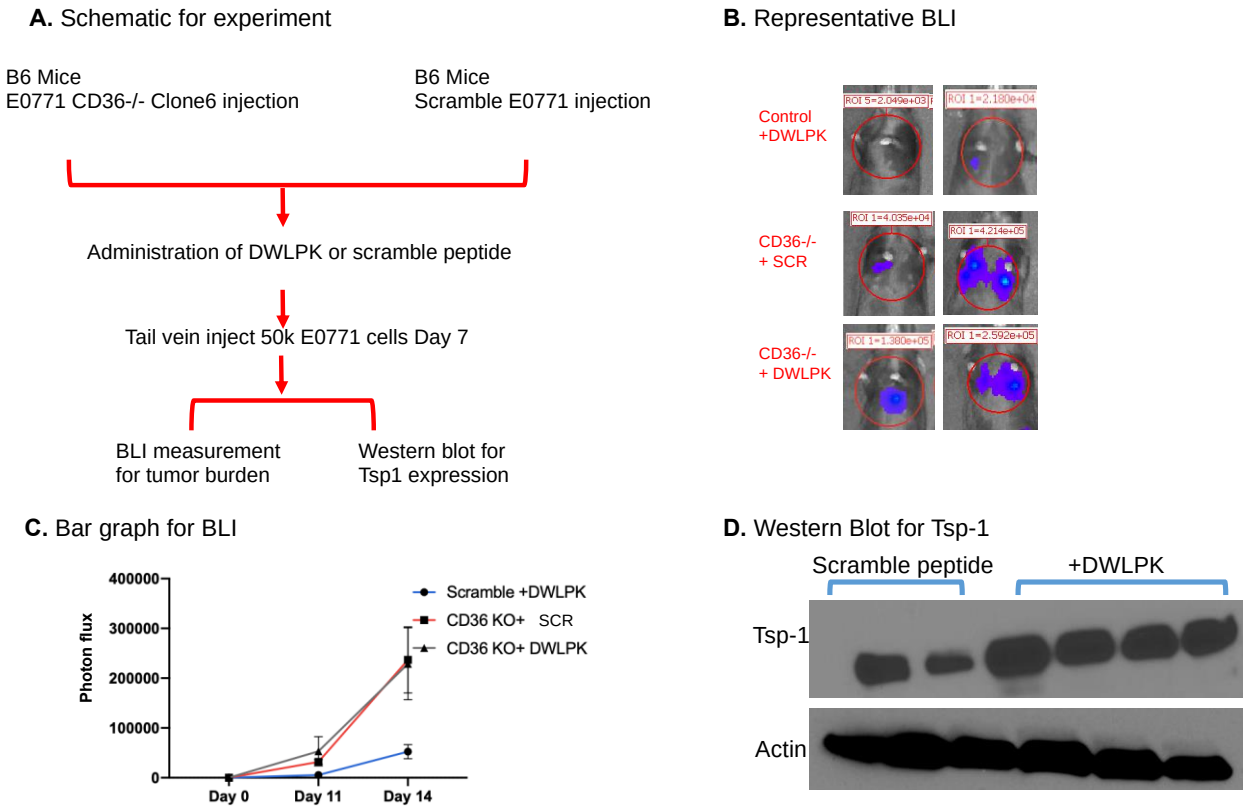

Figure 14. A) Schematic for CD36KO vs. control EO771 tumor cells injected tail vein in DWLPK- or scrambled peptide (vehicle)-treated mice. B) Representative lung BLI images for different treatment groups. C) Quantification of lung BLI. D) Protein expression of Tsp-1 in lung lysates of mice treated with scrambled vs DWLPK peptide.

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?

Year 1 (2016-2017)

Dr. Mittal has given invited seminars on this topic:

1) American Association of Cancer Research Annual Meeting April 2017
2) University of Nebraska Medical Center Feb 2017
3) University of Missouri March 2017
4) Columbus, Metastasis symposium, Cyprus Nov 2016

We published the paper that was used as preliminary data for this DOD grant:

Tina El Rayes, Raul Catena, Sharrell Lee, Marcin Stawowczyk, Natasha Joshi, Claudia Fischbach, Charles A. Powell, Andrew J. Dannenberg, Nasser K. Altorki, Dingcheng Gao and **Vivek Mittal**. Lung inflammation promotes metastasis through neutrophil protease-mediated degradation of Tsp-1. **Proc Natl Acad Sci U S A.** 2015 Dec 29;112(52).

This paper described the modified DWLPK pepide that will be used in Aim 3 of the proposal:

Wang S, Blois A, El Rayes T, Liu JF, Hirsch MS, Gravdal K, Palakurthi S, Bielenberg DR, Akslen LA, Drapkin R, **Mittal V**, Watnick RS. Development of a therapeutic cyclic peptide that targets ovarian cancer via the tumor microenvironment (2016) **Sci Transl Med**. 2016 Mar 9; 8(329): 329ra34

Year 2 (2017-2018)

Dr. Mittal has given invited seminars on this topic:

1) CSBC Annual Meeting – Broad Institute, Cambridge, MA Oct 2017
2) TEMTA Meeting MD Anderson Cancer Center, Houston, TX Dec 2017
3) Sylvester Cancer Center Miami, FL Dec 2017
4) PSOC Annual Retreat Ithaca, NY Jan 2018
5) IUMB Symposium Seoul, Korea June 2018
6) CBSC Symposium, Washington DC Sept 2018

Year 3 (2018 – 2019)

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)

NCE Year (2019 – 2020)

Dr. Mittal and the post-doc Dr. Ramchandani were invited for in-person and virtual seminars and poster presentations on this topic at the following symposia:

1) NCI PSOC Annual Retreat Ithaca, NY Feb 2020 (poster & seminar)
2) Cold Spring Harbor Labs' Mechanisms & Models of Cancer Virtual Meeting, Cold Spring Harbor, NY August 2020 (invited talk)
3) Stony Brook University's Institute of Chemical Biology & Drug Discovery Annual Virtual Symposium, Stony Brook, NY September 2020 (invited talk)

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

N/A

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. In the NCE year, we were able to further characterize the TSP1-CD36 axis in the metastatic phenotype, administer the E0771 TNBC cells and Psap peptide in KO models, and test the combined efficacy of the peptide and sivelestat in the inhibition of angiogenesis and metastasis. Were also able to combine the two clones together for guide #4, #6, and scrambled to develop polyclonal KO and scr cells.

These studies represent 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 laboratory shutdown due to the COVID-19 pandemic from March – June 2020 caused many delays in experiments. The subsequent restart of research activity in July 2020 was slowed by the 50% capacity requirement of all research staff in order to maintain social distancing and prevent any viral spread. At the completion of the NCE period on 09/29/2020, we were still operating at a 50% capacity level.

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) – 35% effort*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4.2*
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?

Dr. Mittal received a two-year research grant W81XWH-19-1-0286 from the United States Dept of Defense CDMRP entitled, "Targeting tumor intrinsic immunosuppressive mechanisms to enhance efficacy of immune checkpoint blockade in lung cancer." Dates are from 08/01/2019 – 07/31/2021. He is spending 10% effort (1.2 calendar months).

Dr. Mittal received a two-year research grant UG3CA244697 from the NIH NCI entitled, "Intercepting progression from pre-invasive to invasive lung adenocarcinoma." Dates are from 09/25/2019 – 08/31/2021. He is spending 10% effort (1.2 calendar months).

What other organizations were involved as partners?

None

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

9. **APPENDICES:**
   Nothing to report