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TITLE: Characterization of Clustered CTCs to Eliminate Breast Cancer Metastasis

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14. ABSTRACT
This program examines the role of platelets and IL11 in breast cancer tumor cell clustering and metastasis in the lung. In year 1, we determined that only 5 days of IL11 treatment to C57BL/6mice resulted in a 2-3-fold increase in platelet counts. In year 2, we created a murine model for metastatic breast cancer in C57BL/6 mice and established facilities for the studies after a collaborator left. In year 3 we characterized the labeled E0771.LMB cells for these investigations. By cell sorting, we have prepared a homogenous population of cells with a high degree of Td-tomato expression (an internal marker of the luciferase reporter) that correlates with the degree of bioluminescence measured. With these cells, we now show critical proof of concept studies that platelet number alone greatly influences the degree of breast cancer tumor cell metastasis. Mice, made thrombocytopenic with anti-CD41, have less breast cancer cell seeding, smaller lung metastasis and fewer metastatic foci than mice with a normal platelet count. These studies prove the importance of platelet number in breast cancer metastasis and indicate that number alone at time of seeding influence metastasis..

15. SUBJECT TERMS
IL11, CD49b, CD44, miR30c, platelets, breast cancer stem cells, circulating tumor cells, CD41

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TABLE OF CONTENTS

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

Background: Circulating tumor cells (CTCs) with stem cell properties are considered the seeds of distant metastasis. The mechanisms how CTC clusters are generated are unclear. We aimed to determine if CTC clustering with lung metastasis is enhanced by platelets. Our aim is to examine the participation of platelets, both number and function on the IL-11/CD49b in the pathway for CTC/platelet clusters. We will examine the role of platelet number and function, including IL11 and CD49b, with CTC clusters.

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

Breast cancer stem cells; circulating tumor cells. IL11; CD49b; CD44; miR30c; platelets; thrombocytopenia; thrombocytosis, CD41

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Does IL11 promote CTC clustering and polyclonal metastasis in vivo. (1-4 months) – partial completion

Task 2: Are platelets activated by IL11 to cluster with CD44+ CSCs/CTCs in vitro and in vivo? (4-8 months) – partial completion

Task 3: Is CD44 regulated by and required for IL11 function in promoting CSC/CTC cluster formation? (8-12 months) – partial completion.

Task 4: Does IL11 induce CD49b expression in platelets and BCSCs? – not done yet

Task 5: Is CD49b important in IL11 function in CSC/CTC clustering? – not done yet

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Our program is to examine the influence of IL11 on platelets and their combined effect on breast cancer tumor clustering and metastasis to lung and other organs. In the first year of the award, we learned that only C57CL/6 mice, not SCID or balbc, appeared to be responsive to IL11 with a 2-3 fold increase in platelet count. Thus, we needed to establish a syngeneic, C57BL/6 murine model for breast cancer metastasis.

The literature provided a guide for such a model using EO771.LMB cells, a C57BL/6-mouse-derived model of spontaneously metastatic mammary cancer (Johnstone CN *et al*. Disease Models & Mechanisms (2015) 8:237. EO771.LMB tumors are derived from poorly metastatic parenteral EO771 mammary tumors. EO771.LMB cells were purchased from the ATCC. The cells are positive for EGFR and a mutant p53.

Several operational issues, however, presented itself upon the initiation of this work and it interfered with the research progress of the program in year 2. (1) My Co-PI and collaborator on the DOD grant for the murine model studies, Dr. Huiping Liu, left Case Western Reserve University and transferred her project and support to Northwestern University in the late Spring, 2017. It has been originally planned that the murine metastasis models were to be performed in her laboratory. (2) I needed to submit to the IACUC the protocols for murine breast cancer metastasis that are novel for my laboratory to IACUC. The submission of these new protocols for the murine breast cancer metastasis to IACUC occurred at the same time that I needed to renew (done every 3 years) my entire animals' protocol at Case Western Reserve University. (3) Since we were using transfected cells, the actual protocol also had to be reviewed and approved after the Animals' Protocol approval by the CWRU Institutional Biosafety Committee (IBC). (4) Once CWRU IACUC and IBC approvals were completed, DOD asked for an additional complete review of the newly approved Animals' Protocol from CWRU. During the time of when the institutional approvals were taking place (June 2017 through March 2018), NO ANIMAL RESEARCH ON THIS PROJECT COULD BE PERFORMED. Thus, there was a MAJOR DELAY in getting this project started.

However, in the Spring of 2018, we obtained luciferase-labeled E0771.LMB cells that were stably transfected via a lentivirus to express luciferase-2-tdTomato (L2T). The Td tomato marker in the expression vector has proven to be a useful marker for the labeled cells (see below). Preliminary experiments were presented in the Year 2 Progress Report. It can be clearly stated that at this juncture, that critical experiments have been performed to indicate the role of platelets in promoting circulating tumor cell metastasis. Suffice-it-to-say, platelets have critical role in cancer cell metastasis. Thrombocytopenia reduces cancer cell metastasis.

The following major sections will be organized as follows:

1. Characterization of the E0771.LMB cells for investigation.
2. Characterization of CD44 and CD49b on the E0771.LMB cell line.
3. Characterization of IL11 and CD41 on platelet counts.
4. Influence of platelet count at the time of tumor cell injection on extent of tumor metastasis as determined by bioluminescence and number of metastatic sites.

The following sections addresses each of these categories.

1. **Characterization of the E0771.LMB cells for investigation**.

Before presenting our proof of concept investigations on the role of platelets in cancer metastasis, we performed further characterization of the labeled E0771 LMB cell line that we will be using for our investigations. We wanted to be certain about the homogeneity of the cell preparation. Flow cytometry was performed to sort the labeled cells by Td-tomato to recognize and grow only those cells transfected with the luciferase expression system. This work is characterized in **Fig 1**.

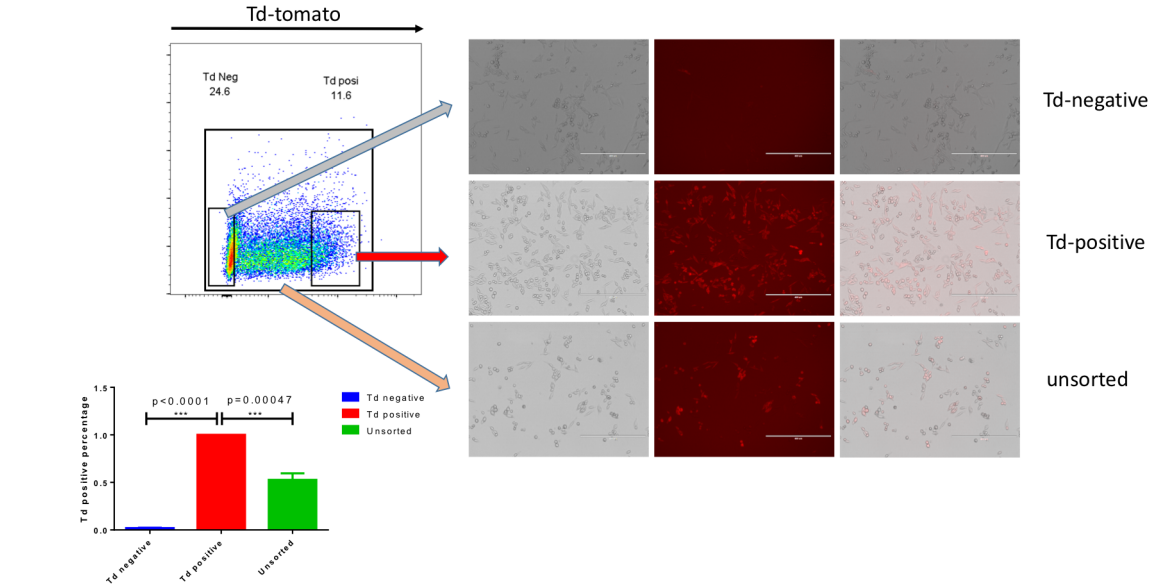

Fig 1. *E0771.LMB cell sorting.* The transfected cells were sorted by flow cytometry. Upper left panel: showing Td-negative and Td-positive cells. Large middle panel: phase contrast, Td-tomato, and combined columns (left to right) for Td-negative (top), Td-positive (middle) and unsorted (bottoms) rows. Lower left panel: % Td positive cells in each group (Td-negative, Td-positive, and unsorted.

As seen in **Fig 1** (Upper left panel) our labeled cells sorted into 3 populations, Td-negative, Td-positive, and unsorted. These sorted cells are shown in the middle panel where the density of Td tomato expression is indicated. Td-positive cells on phase contrast, immunofluorescence, or combined has the highest number of cells (middle row of major graphic above). Alternatively, both Td-negative and unsorted cells had no or little Td tomato expression (top and bottom rows on major graphic above). As shown in the lower left panel, the Td-positive cells sorted into a more homogenous population. These efforts have resulted in a more homogenous population of cells for investigation.

Additional investigations next determined if the degree of Td tomato expression (i.e. the most homogenous population of vector expressing cells) in the population of Td-positive cells correlates with measured bioluminescence (**Fig 2**). As shown in **Fig 2**, upper left panel, there is an

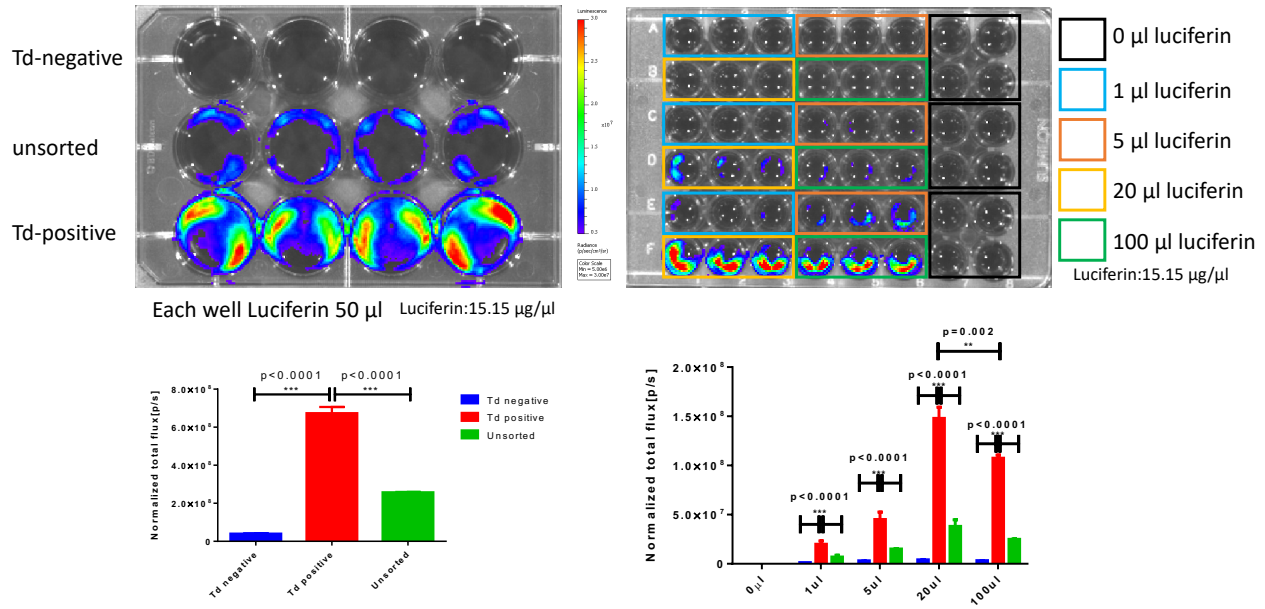

Fig 2. *Comparison of Td tomato expression and bioluminescence.* Upper left panel. Constitutive expression of bioluminescence among Td-negative, unsorted, and Td-positive cells by bioluminescence. Upper right panel: the influence of increasing concentrations of luciferin (0-100 µl, 1 ml=15.15 µg) on bioluminescence. Rows A and B: Td-negative cells; Rows C and D: unsorted cells; Rows E and F: Td-positive cells. Lower left panel: normalized total bioluminescence among the 3 groups of cells (flux=bioluminescence). Lower right panel: normalized total bioluminescence (flux) at each luciferin concentration (0 to 100 µl).

extraordinary difference in the degree of bioluminescence among Td-negative, unsorted, and Td-positive cells. As shown in **Fig 2**, lower left panel, the degree of bioluminescence in the Td-positive cells is highly significantly different in the Td-positive cells vs either the Td-negative or unsorted cells. Note: on the ordinate of the figure, bioluminescence is expressed as "flux". **Fig 2,** upper right panel, the degree of luminescence was examined based upon the degree of luciferin (substrate for luciferase) added to the cells cultured in the wells. Note 1 µl equals 15.15 µg luciferin. As can be seen in the figure, 1-100 µl of luciferin produced no luminescence in Td-negative cells (Rows A and B). Unsorted cells have an inkling of positive bioluminescence when 20 to 100 µl of luciferin is added (Rows C and D). Alternatively, the Td-positive cells show some luminescence at all amounts of luciferin added that maximizes at 20 µl added substrate (Rows E and F). In **Fig 2**, lower right panel we show that at any amount of added luciferin, the degree of luminescence (flux) in the Td-positive cells is significantly greater than that seen in Td-negative and unsorted cells, respectively. The combined data in **Fig 1** and **Fig 2** indicate that sorted Td-positive cells that will be used in subsequent experiments will be a homogenous population of luciferase expressing cells and the vector marker, Td tomato, correlates with the degree of bioluminescence expressed in the cells.

2. Characterization of CD44 and CD49b on the E0771.LMB cell line.

Investigations next characterized the E0771.LMB cells for the expression of CD44 and CD49b. In previous investigations, we characterized CD44 and CD49b on the membrane of human

platelets. IL11 and the IL11R were not present on platelet membranes. Using flow cytometry, we determined if CD44 and CD49b are also expressed on the membrane of E0771.LMB cells (**Fig 3**).

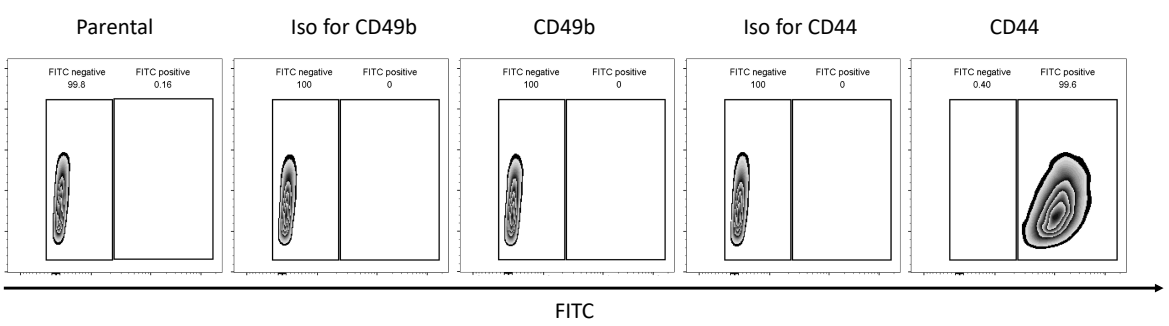

Fig 3. *CD44 expression on E0771.LMC cells.* E0771LMC cells were examined for membrane expression of CD49b and CD44. A negative flow cytogram are data left of the dividing line. A positive flow cytogram are data to the right of the line. "Iso" represents an immunoglobulin of the same subtype as the antibody directed to the epitope of CD44 or CD49b. The flow cytogram labeled "Parental" are cells alone without added ab.

As shown in **Fig 3**, the CD49b epitope was not observed on the membrane of the E0771 LMC cells. Alternatively, CD44 epitope is expressed on the external membrane of E0771.LMC cells. These data indicate that CD44 is expressed on both platelets (Year 1 progress) and E0771.LMC tumor cells (Year 3 observation). CD49b is only found on platelets.

3. Characterization of IL11 and CD41 treatment on platelet counts.

a. Studies with IL11

One of the main original goals of this program is to determine the influence of IL11 on platelets and circulating tumor cell clustering. Our previous investigations did not demonstrate IL11 or its receptor on the platelet membrane. However, we do know that in humans, IL11 treatment was one of the first agent recognized to elevate platelet counts (Neben TY et al. Blood 81:901, 1993). Since the mature murine platelet does not have the IL11R, this effect must be occurring at the level of the megakaryocyte.

Preliminary experiments were determined if IL11 at 75 μg/kg sc daily increased murine platelets counts and E0771.LMC cell metastasis. Platelet counts were obtained pre-treatment and 7 days after treatment. Also, at 7 days of treatment, 3 X 10^5 E0771.LMC cells were injected using a 30-gauge needle in the tail vein of female C57BL/6 mice. The results of this experiment, however, were negative. First, IL11 treatment after 7 days did <u>not</u> increase the treated mice platelet count. Second, at 28 days, IL11-treated mice did <u>not</u> have increased lung metastasis.

Analysis. These experiments with IL11 were preliminary. A negative experiment could have arisen for several reasons. 1) we did not know if the recombinant IL11 used in the experiment were functional. It was a reagent that had been frozen at -80^{o}C for 1.5 years and we did not have an established assay when reconstituted if the agent were functionally active. 2) the dosage used (75 μg/kg sc daily) was half of a previously effective dose that had been published nearly 25 years ago. Differences in preparation, specific activity, and purity could account for activity differences. More studies with IL11 are needed.

Further investigations are needed to examine the role IL11 in platelet-tumor cell interactions in metastasis. Although there are several reasons why the IL11 experiments were negative, we

decided to put those experiments aside for the time being to examine a first principle of these investigations, i.e. what is the role of platelet number at the time of tumor cell metastasis.

b. Studies with anti-CD41

Investigations next were performed to determine the role of platelet number at the time of tumor metastasis on the extent of future tumor seeding and tumor cell growth of the metastatic tumor growth. CD41 is the platelet integrin alpha 2b from the gene ITGA2B. It is one integrin in the platelet glycoprotein GP2b3a or $\alpha_{2b}\beta_3$ integrin, the major aggregation receptor and binding site for fibrinogen and the other adhesive glycoproteins, fibronectin, thrombospondin, and von Willebrand factor. Heterologous antibody to CD41 induces thrombocytopenia in mice (**Fig 4**).

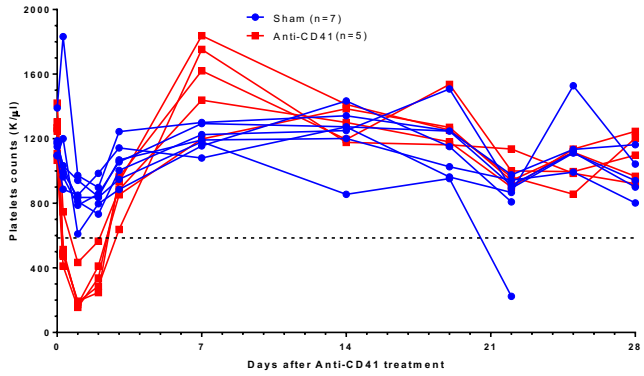

Fig 4a. *Platelet count of mice after anti-CD41 infusion.* In these experiments, mice were injected with 10 µg anti-CD41 ab/mouse and the platelet counts were measure at the times indicated. Sham injections are PBS.

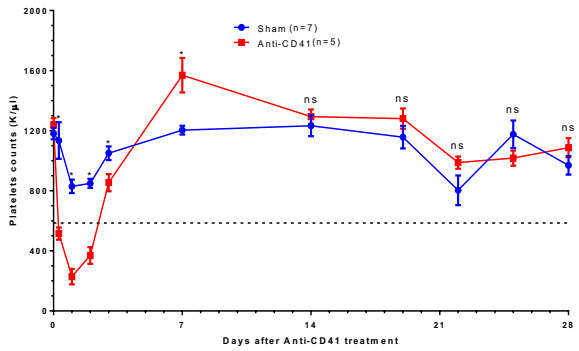

Fig 4b. *Mean platelet count in mice after anti-*CD41 antibody (red curve, n=5) versus Sham injection of PBS (blue curve, n=7). The data are mean +/- SEM.

Fig 4a shows individual platelet counts for 5 mice treated with antibody to CD41 and 7 Sham antibody-treated (PBS) mice after 28 days. **Fig 4b** shows mean $\pm$ SEM of 5 anti-CD41- and 5 Sham antibody-treated mice. Both figures show that the individual and mean nadir after antibody treatment is 24 h. The peak of anti-C41 treatment to induce thrombocytopenia is 24 h, where there is at least a 85% reduction in platelet count at that time.

4. Influence of platelet count at the time of tumor cell injection on extent of tumor metastasis as determined by bioluminescence and number of metastatic sites.

Studies next examined the influence of platelet count at the time of tumor metastasis on subsequent seeding and tumor cell volume and number of metastatic sites. In this model, tumor metastasis is experimentally induced by injection of 3 X 10^5 E0771.LMC cells in the tail vein of female C57BL/6 mice. In these experiments, we exclusively used female mice because we are working with a murine breast cancer model. Since IL11 treatment has not to date been shown to be effective in increasing platelet count of host mice, we chose to perform this proof-of-concept experiment by making a group of mice thrombocytopenic after treatment with anti-CD41 vs control. Using the data in **Fig 4** above, all mice receiving anti-CD41 were shown to be at their platelet nadir at 24 h. At that time, both anti-CD41- and Sham-treated mice were injected with 3 X 10^5 E0771.LMC cells in the tail vein. Bioluminescence measurements of any tumors developing in the apices of the lung were performed from Day 7 to Day 28. Note: from day 14 to 28, mice were scanned every 3 days since some animals die if their tumor burden becomes too large. At Day 28, all surviving mice are scanned and then euthanized. The results of these experiments are shown below. Studies also

were performed to show the influence of thrombocytopenia produced by anti-CD41 on tumor cell metastasis and tumor growth over 28 days. As seen in **Fig 5**, there appears to be a significant

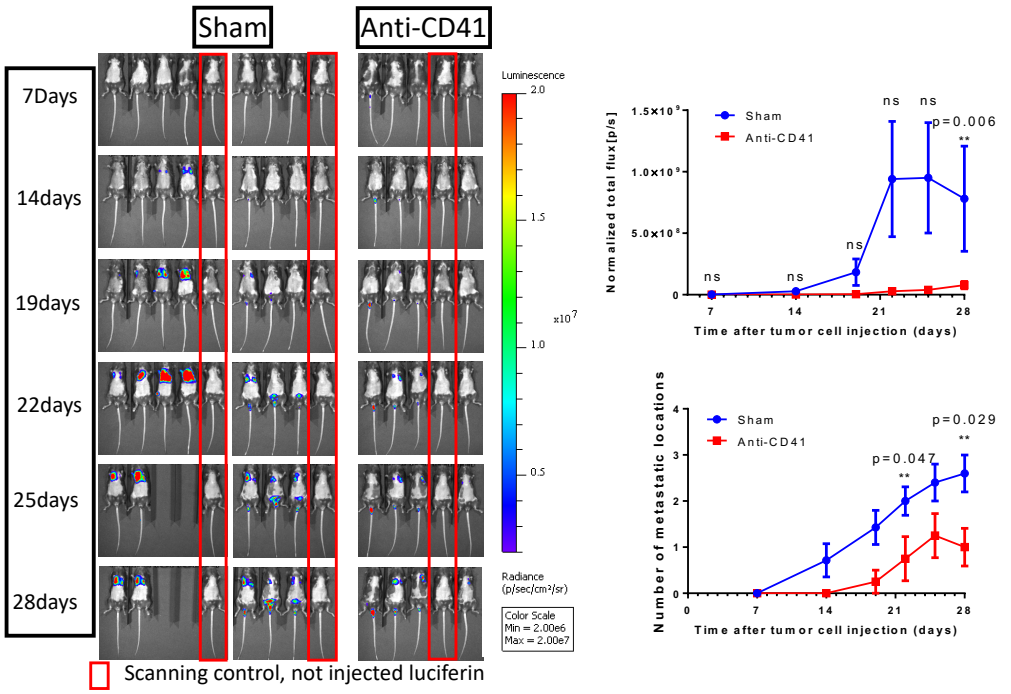

Fig 5. *Tumor bioluminescence signal in mice over 28 days after tumor cell injection.* Panel on left. This panel shows bioluminescence scans after injection of luciferin. The mice were scanned in groups of five and each has a negative control, i.e., a mouse not injected with luciferin. The animals in the red box are the negative controls. The absent lanes at days 25 and 28 are positions of previous mice that died. The absent lanes were not included in the calculations at days 25 and 28. Panel on the upper right. This graph shows change the value of bioluminescence of the anti-CD41- or Shan-treated mice over the time of the experiments. P values were determined by group-paired t test. The term "flux" means bioluminescence. Panel on the lower right. This graph shows the number of metastatic sites detected over time in the anti-CD41- and Sham-treated mice. P values were determined by group-paired t test.

difference after 14 days in the number and size of the bioluminescence in the lung in Sham-treated mice versus anti-CD41-treated mice. Once can see this visually in the Panel on the left. When the luminescence signal is normalized and plotted as bioluminescence (flux) versus time (Panel on the upper right), there is a large difference in the Sham versus anti-CD41 curves. The two groups do not achieve significance by paired T testing until day 28. However, when the number of metastatic foci were counted from the bioluminescence scans, there is a clear separation of significance difference at Day 22 (Panel on the lower right). These data indicate that making mice thrombocytopenic with a > than 85% reduction in their platelet count reduces the degree of metastasis at the time of tumor cell infusing.

The findings above indicate a fundamental point that validates this project and the planned future experiments we have. Platelet number influences the degree of tumor cell metastasis at the time of seeding. Thrombocytopenia reduces tumor cell metastasis. These data indicate that platelet number alone is important for tumor cell metastasis. This observation will be the basis for all out future investigations.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

First, I had decided that this project needs a "professional" full-time associated with it. In order to successfully develop and complete this project, I have recruited a PhD candidate from the Department of Pharmacology, Mr. Peng Zeng, to take the lead in the development of this project. He has taken charge of the project and the presentation in this report is the result of his efforts. This project will be the basis of his PhD work here at Case Western Reserve University.

Second, we have begun to develop new techniques such as cell sorting using Td tomato and tumor cell size measurement and number by luciferase-induced bioluminescence. We expect other new laboratory technologies to develop as result of this project.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

We now have enough data to 1) have the graduate student present this work to his PhD committee and 2) begin to prepare local presentation to his fellow graduate studies and in conferences in our research and clinical cancer center.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We intend to focus efforts on the "platelet number" story of the program. First, we will confirm that thrombocytopenia reduces cancer cell metastasis by inducing thrombocytopenia with antibody to platelet GPIbα. Next, we plan to show that thrombocytosis leads to increased cancer cell metastasis. We will increase platelet number by rebound after induced thrombocytopenia, romiplostim, and IL11. We next will determine what platelet receptors contribute to cancer cell metastasis. We will determine if nanoantibodies to GP2b3a or GP1bα and if antibodies to CD44 or CD49b induce thrombocytopenia. If not, we will determine if blocking those receptors, independent of platelet number, reduce cancer cell metastasis. Finally, if we determine a receptor(s) to be involved with metastasis, we will obtain its genetic knockout for further studies.

4. **IMPACT:** Describe 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

This work may turn out to be high impact. In clinical oncology, efforts are underway to see if platelet growth factors can be used to increase platelet counts to allow for planned chemotherapy administration. If elevated platelet counts increase metastasis, this effort to raise platelet counts for chemotherapy administration may not be attractive.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

This work may have impact on clinical hematology and oncology practice. It has implications for thrombosis as well.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report yet

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

No changes in planned protocol to date

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

At present, no anticipated delays. Since DOD financial support will be completed, we will move this project along with other funds in the short run. I will by applying for more DOD funds as the project develops.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Since my collaborator has left, there are been a significant increase in costs that were not anticipated when the project was submitted. The mouse expenses were not included in my budget and each experiment that runs over 6 weeks is a significant cost outlay for mice. These costs may be a problem in the near future.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not relevant for this project

Significant changes in use of biohazards and/or select agents

Not relevant for this project

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding,

prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Dr. Alvin H. Schmaier MD
Project Role: PI
ORCID ID: 0000-0002-3884-6234
Nearest person month worked: 1 month
Contribution to the project: Designed the studies, trained the Research Assistant, analyzed the data; wrote the grant and progress report.

Alona Merkulova MS
Project Role: Research Assistant IV
ORCID ID: none
Nearest person month worked: 7 months
Contribution to Project: Performed many experiments, animal care, obtained the final data for analysis; responsible for all activities related to this program.
Funding Support: This award and NHLBI HL144113

Peng Zeng
Project Role: Graduate Student
ORCID ID: 0000-0002-6394-3589
Nearest person month worked: 3.5 months
Contribution to Project: Performed cell sorting, mouse platelet counts, and bioluminescence experiments
Funding Support: NCI CA223301

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Active Support.

BC150596P1, Schmaier (PI) 06/01/16-5/31/20 0.32 Calendar mo
CDMRP, Department of the Army
Characterization of Clustered CTCs to Eliminate Breast Cancer Metastasis
This program seeks to characterize the role of platelets in participating in breast cancer metastasis. It is a novel project that will examine the roles of IL11, CD49b and CD44 in platelets and circulating breast cancer cells to determine if these cells conjoin to promote metastasis. There is no overlap with the current proposal.
Total Support: $317,359; Direct Support: $200,247.

R01 HL126645-01, Simon (PI) 1 2/01/15-11/31/20 1.2 Calendar mo
NIH/NHLBI
"MRP-14, CD36 and Thrombosis"
The overall objective of this proposal is to define the role of MRP-14 in vascular inflammation and thrombosis.
Role: Co-PI
Total Support: Salary support only

R01 AI130131-01 (Kazura, PI) 04/01/17-3/31/22 1.2 Calendar Mo
NIH/NAI
"*Kruppel-Like Factor 2 Counters Vascular and Immunologic Dysfunction in Child Cerebral Malaria*"
The overall goal of this program is to examine how the head domain of the malaria parasite influences the constitutive anticoagulant nature of vascular endothelium.
Role: Co-PI
Total Support $70,876; Direct Support $44,717.

Shire Investigator-Initiated Support (Schmaier, PI) 4/1/2018-3/31/2019 0.32 Calendar Mo.
"*Prolylcarboxypeptidase Activates Prekallikrein.*"
This project critically examines if endothelial cell prolycarboxypeptidase has the ability to activate PK to plasma kallikrein to generate bradykinin and factor XIIa. We examine how ambient C1 inhibitor levels influence PRCP activation of PK and indirectly FXII.

Total Support: $93,331 for 1 year. Direct Support $74,663.

R01 HL144113 (McCarty/Hinds) 07/01/18 – 06/30/23 1.8 Calendar mo.
NIH GM
Title: *Contact Pathway Activation on Vascular Devices*
Goal: The goal of the project is to define the therapeutic potential of targeting contact activation to prevent vascular device-related thrombosis through the use of in vitro, ex vivo and in vivo models of vascular devices.
Total Support: $103,374; Direct Support $64,609.

R21 CA223301-01A1, Schmaier (PI) 12/01/18-11/30/20 1.2 Calendar mo
NIH
"Ponatinib Induces Vascular Events in CML-Mechanisms and Correction"
The goal is that ponatinib's pharmacologic inhibition of vascular ABL1 kinase and platelet p-Lyn results in reduced antithrombotic vascular function and hyperactive platelets, leading to heightened arterial thrombosis.
Role: PI
Total Support: $187,646; Direct Support: $117,279

U01 HL143402-01, McCrae (PI) 7/01/18-06/30/23 1.2 Calendar mo
NIH
"Novel approaches to improve prediction of cancer-associated thrombosis". This program examines contact activation mechanisms as contributors to thrombosis in cancer.
Role: Co-I
Total Support: $82,328; Direct Support: $51,455.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

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. 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.

ABSTRACT. In year 3 of this award we have securely grounded this project in evaluable data that indicate that this work is an important project to understand mechanism(s) for breast cancer metastasis. We have developed a workable model to detect breast cancer metastasis. Using cell sorting, we prepared an enriched population of Td-tomato E0771.LMC cells can be prepared that have a high rate of luminescence. Our studies show that enriched E0771.LMC cells with a high percentage of Td-tomato expression have a correlated high rate of bioluminescence. Addition studies show that mice can be made thrombocytopenic with injection of a heterologous antibody to CD41. After a 10 μg injection of anti-CD41, mice are made thrombocytopenic within 24 h. There is am 85% reduction in their starting platelet counts. If E0771.LMC cells with a luciferase marker are injected (3 X 10^5 cells/ml) into thrombocytopenic mice, there is significantly less tumor seeding and growth in the lungs at 28 days and there are less number of metastatic lesions in the lungs at 22 to 28 days. These data indicate that platelet number at the time of tumor cell injection significantly influences tumor cell seeding and metastasis.