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4. TITLE AND SUBTITLE:
Characterization of Clustered CTCs to Eliminate Breast Cancer Metastasis

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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 IL-11 and CD49b, with CTC clusters.

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

Breast cancer stem cells; circulating tumor cells. IL-11; CD49b; CD44; 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.

The purpose of this proposal was to determine the role of IL-11 in platelet activation as it relates to cancer cell metastasis and is IL-11 activity mediated through CD49b and CD44. The original aims of the proposal were as indicated below.

Aim II. Examine the role of IL-11/ CD49b in CTC/platelet clusters and their signaling pathway.

My role in this program in collaboration with Dr. Huiping Liu was to perform the following studies

Task 2. Are platelets activated by IL-11 to cluster with CD44+ CSCs/CTCs in vitro and in vivo?

Task 3. Is CD44 regulated by and required for IL-11 function in promoting CSC/CTC cluster formation?

Task 4. Does IL-11 induce CD49b expression in platelets and BCSCs?

Task 5. Is CD49b important for IL-11 function in CSC/CTC clustering?

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 was to examine the influence of IL-11 on platelets and their combined effect on breast cancer tumor clustering and metastasis to lung and other organs via platelet CD44 and CD49b. In the first year of the award, we had preliminary data that only C57CL/6, not SCID or balbc, mice appeared to be responsive to IL-11 with a 2-3-fold increase in platelet count. Thus, we needed to establish a syngeneic, C57BL/6 murine model for breast cancer metastasis. This was a major change in initial plans.

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 coincided with the 3-year renewal 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). These renewals were completed by October,2017.
(4) Once CWRU IACUC and IBC approvals were completed, DOD asked for an additional complete review of the entire, 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.

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).

1. Characterization of the E0771.LMB cells for investigation.
We characterized 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 (**Figure 1**, next page)

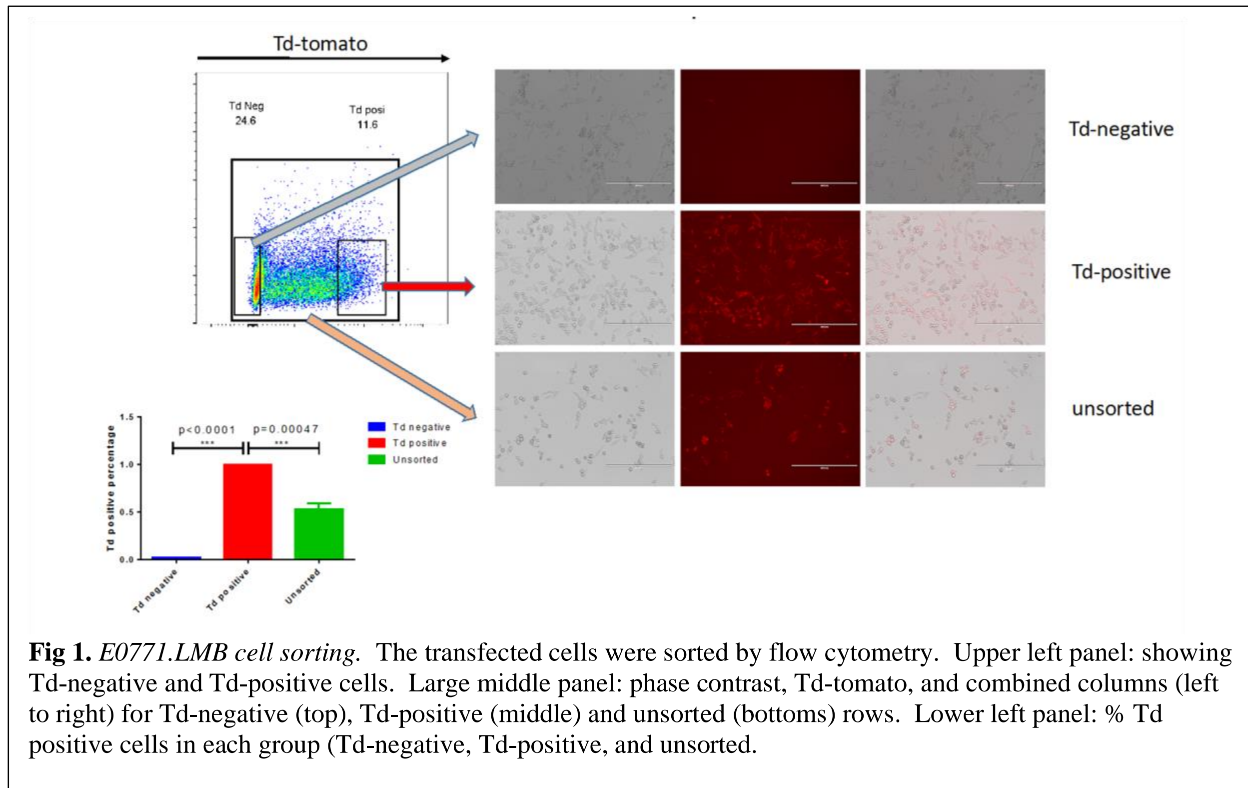

Fig 1. *E0771.LMB cell sorting.* The transfected cells were sorted by flow cytometry. Upper left panel: showing Td-negative and Td-positive cells. Large middle panel: phase contrast, Td-tomato, and combined columns (left to right) for Td-negative (top), Td-positive (middle) and unsorted (bottoms) rows.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

Next, investigations 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 extraordinary

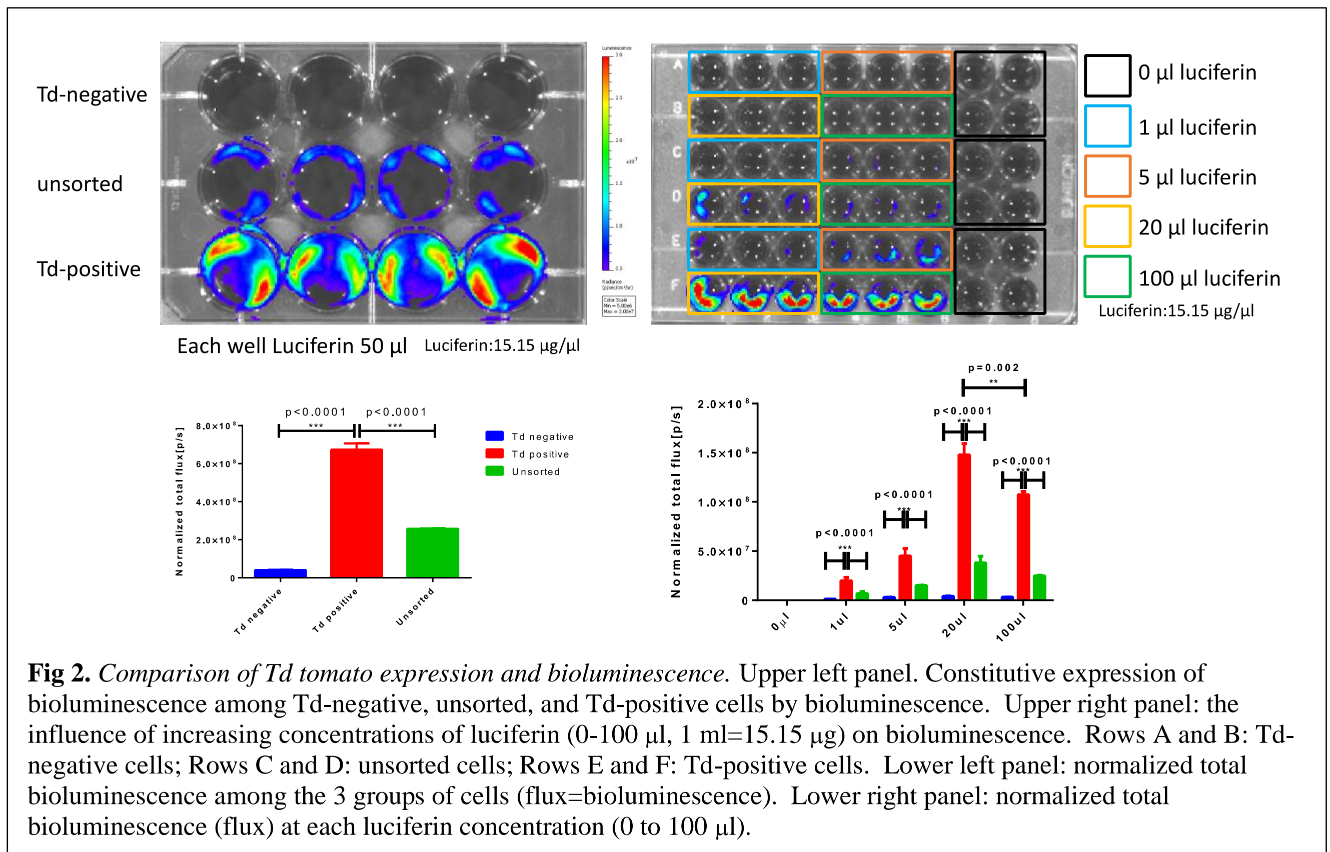

Fig 2. *Comparison of Td tomato expression and bioluminescence.* Upper left panel. Constitutive expression of bioluminescence among Td-negative, unsorted, and Td-positive cells by bioluminescence. Upper right panel: the influence of increasing concentrations of luciferin (0-100 µl, 1 ml=15.15 µg) on 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).

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. Obviously, the presence of these receptors on platelets is essential for them to be able to cluster

with platelets to make circulating breast cancer cells. Using flow cytometry, we determined if CD44 and CD49b are expressed on the membrane of E0771.LMB cells (**Fig 3**). As shown in **Fig**

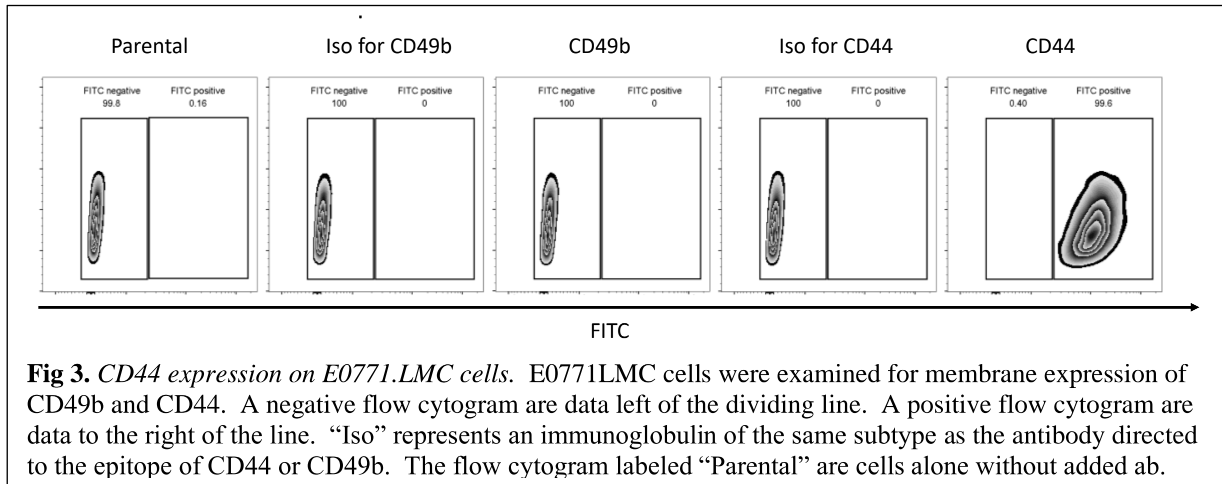

Fig 3. *CD44 expression on E0771.LMC cells.* E0771LMC cells were examined for membrane expression of CD49b and CD44. A negative flow cytogram are data left of the dividing line. A positive flow cytogram are data to the right of the line. "Iso" represents an immunoglobulin of the same subtype as the antibody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

3, the CD49b epitope was <u>not observed</u> on the membrane of the E0771 LMC cells. Alternatively, CD44 epitope is expressed on the external membrane of E0771.LMC cells. Therefore, E0771 LMC cells can only participate in clustering with platelets via CD44, not CD49b.

3. Characterization of IL-11, IL-11R, CD44 and CD49b on platelets.

Investigations sought to determine if the above proteins were on platelets. As shown in **Fig 4**, antibody to IL-11r (bottom left) did not detect any IL-11R antigen on the surface of washed, non-activated platelets. These data are similar to the IgG antibody control (top left) for the antibody to IL-11r studies. Alternatively, antibody to CD44 (bottom, center) shows robust antigen expression (73%) on the membrane of unstimulated platelets when compared to its IgG control (top, center). Likewise, antibody to CD49b (bottom, right) was highly expressed (70%) on the membranes of platelets constitutively. This finding is distinct from its IgM control (top, right) that is not expressed on platelet membranes at all. These data indicate that CD44 and CD49b are on the membrane of human platelets. However, IL-11R is not present on platelet membranes and therefore IL-11 does not have a direct effect on circulating platelets.

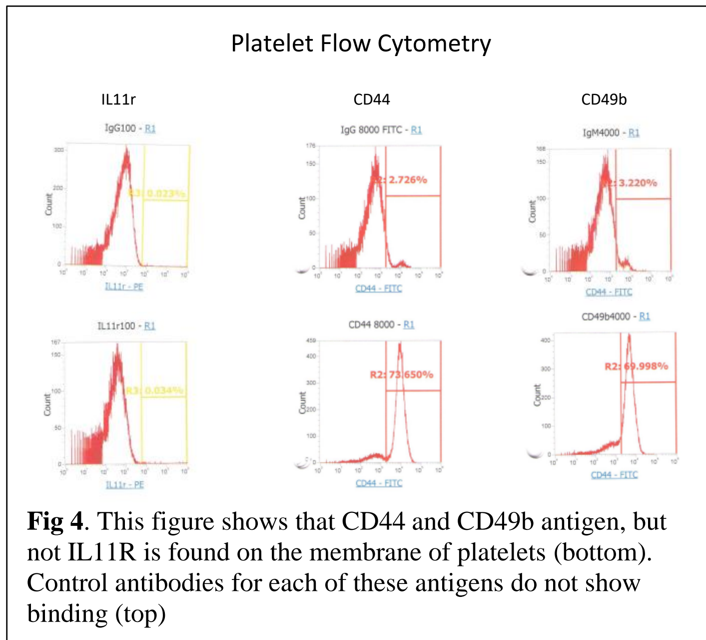

Fig 4. This figure shows that CD44 and CD49b antigen, but not IL11R is found on the membrane of platelets (bottom). Control antibodies for each of these antigens do not show binding (top)

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. IL-11 cannot directly interact with platelets.

4. Characterization of IL-11 and CD41 treatment on platelet counts.

a. Studies with IL-11

One of the main original goals of this program is to determine the influence of IL-11 on platelets and circulating tumor cell clustering. Our investigations do not demonstrate IL-11 or its receptor on the platelet membrane. However, we do know that in humans, IL-11 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 IL-11R, this effect must be occurring at the level of the megakaryocyte.

Preliminary experiments determined if IL-11 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, IL-11 treatment after 7 days did not increase the treated mice platelet count. Second, at 28 days, IL-11-treated mice did not have increased lung metastasis.

b. Studies with anti-CD41

Investigations next determined 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 5**).

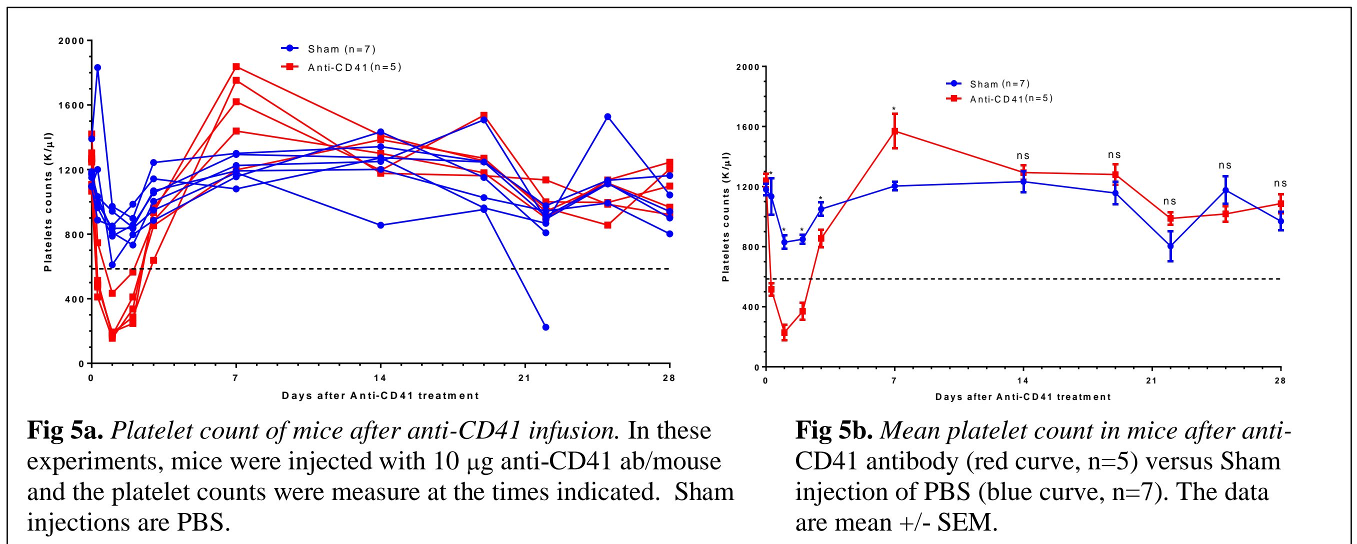

Fig 5a. *Platelet count of mice after anti-CD41 infusion.* In these experiments, mice were injected with 10 μg anti-CD41 ab/mouse and the platelet counts were measure at the times indicated. Sham injections are PBS.

Fig 5b. *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 5a shows individual platelet counts for 5 mice treated with antibody to CD41 and 7 Sham antibody-treated (PBS) mice after 28 days. **Fig 5b** 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 an 85% reduction in platelet count at that time. Thus, anti-CD41 induces initial thrombocytopenia. All platelet counts recover to normal by 14 days.

5. 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 IL-11 treatment is not 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 5** 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 6**, there appears to be a significantly

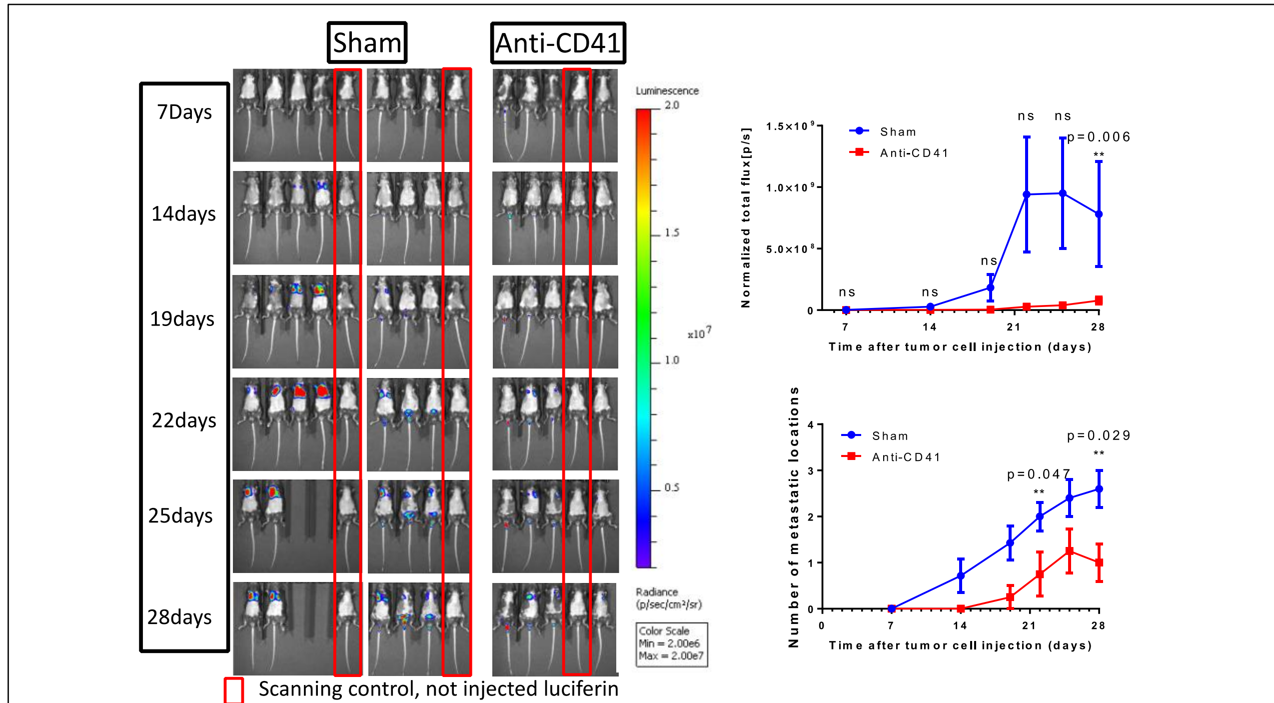

Fig 6. *Tumor bioluminescence signal in mice over 28 days after tumor cell injection.* Panel on left. This panel shows bioluminescence scans after injection of luciferin. The mice were scanned in groups of five and each has a negative control, i.e., a mouse not injected with luciferin. The animals in 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.

reduced tumor cell growth after 14 days as indicated by the number and size of the bioluminescence in the lung in Sham-treated mice versus anti-CD41-treated mice. One can see

this visually in the Panel on the left – overall there is less bioluminescence in the anti-CD41-treated mice than the Sham-treated mice. 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 greater 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 - platelet number at the time of tumor injection influences the degree of tumor cell metastasis at the time of seeding. Thrombocytopenia reduces tumor cell metastasis. These data suggest that platelet number alone may be important for tumor cell metastasis. Further, as shown in **Fig 7**, the reduction of tumor metastasis is associated with increased survival in the C57BL/6 mice used in these studies. Further studies were performed to examine these observations.

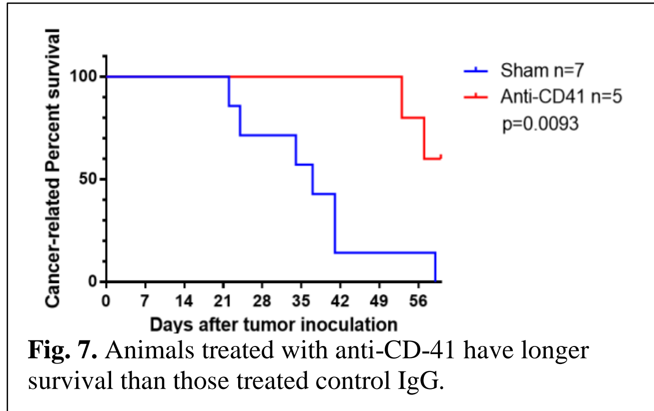

Fig. 7. Animals treated with anti-CD-41 have longer survival than those treated control IgG.

6. What is the influence of platelet CD44 and CD49b on tumor cell metastasis.

Investigations next determined if other antibodies to platelet epitopes also produce thrombocytopenia and this thrombocytopenia is associated with reduced metastasis. Both anti-CD 44 and anti-CD49b were present on the platelet membrane. The appropriate immunoglobulin isotype antibodies did not specifically bind to platelets (**Fig 8, top**). When the anti-CD44 and anti-CD49b antibodies were infused in the mice, they both produced peak thrombocytopenia at 24 h (**Fig 8, bottom left**). Having this information, we embarked upon a similar strategy as we did with anti-CD41 antibody, i.e., inject tumor cells at the platelet count

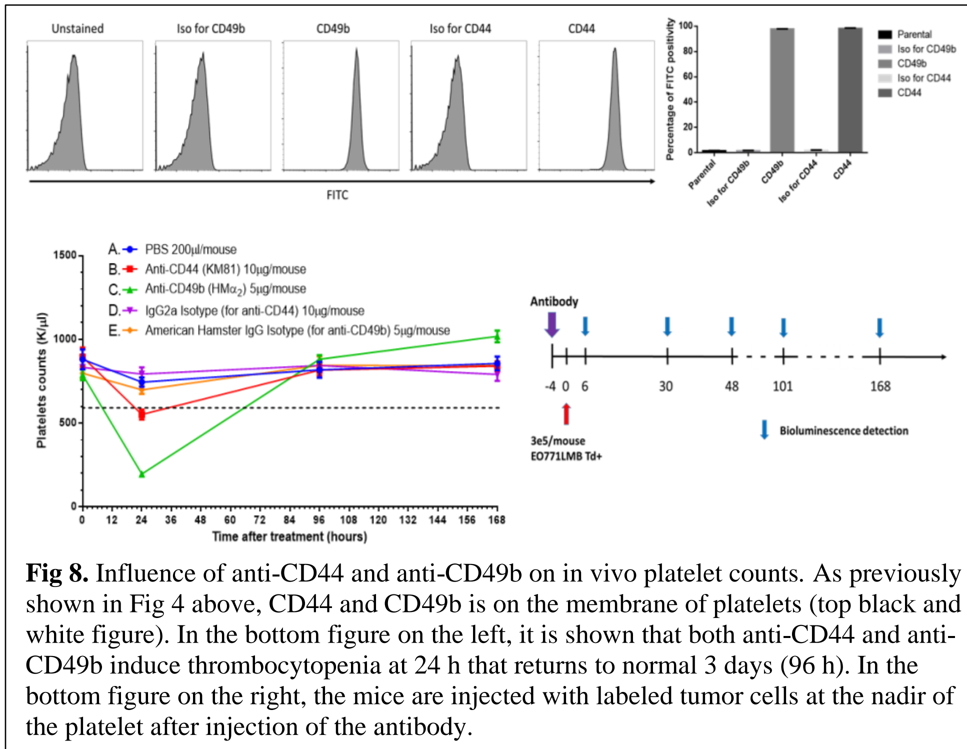

Fig 8. Influence of anti-CD44 and anti-CD49b on in vivo platelet counts. As previously shown in Fig 4 above, CD44 and CD49b is on the membrane of platelets (top black and white figure). In the bottom figure on the left, it is shown that both anti-CD44 and anti-CD49b induce thrombocytopenia at 24 h that returns to normal 3 days (96 h). In the bottom figure on the right, the mice are injected with labeled tumor cells at the nadir of the platelet after injection of the antibody.

nadir to determine if the thrombocytopenia alone at the time of tumor cell injection was sufficient to produce reduced metastasis and increase murine survival after breast cancer cell injection (**Fig 8, bottom right**).

The results of this experiment are shown in **Fig 9**. Infusion of antibody to CD 44 and CD49b made the mice thrombocytopenic. At the time of peak thrombocytopenia, the labeled breast cancer tumor cells were injected into the mice. At both 24 and 28 days, the degree of tumor cell metastasis were the same in the animals that got anti-CD44 and its isotype control and anti-CD49b and its isotype control. These data indicate that thrombocytopenia alone is not sufficient to influence metastatic breast cancer in this murine model. In addition to thrombocytopenia, the epitope of the antibody causing the thrombocytopenia is also important.

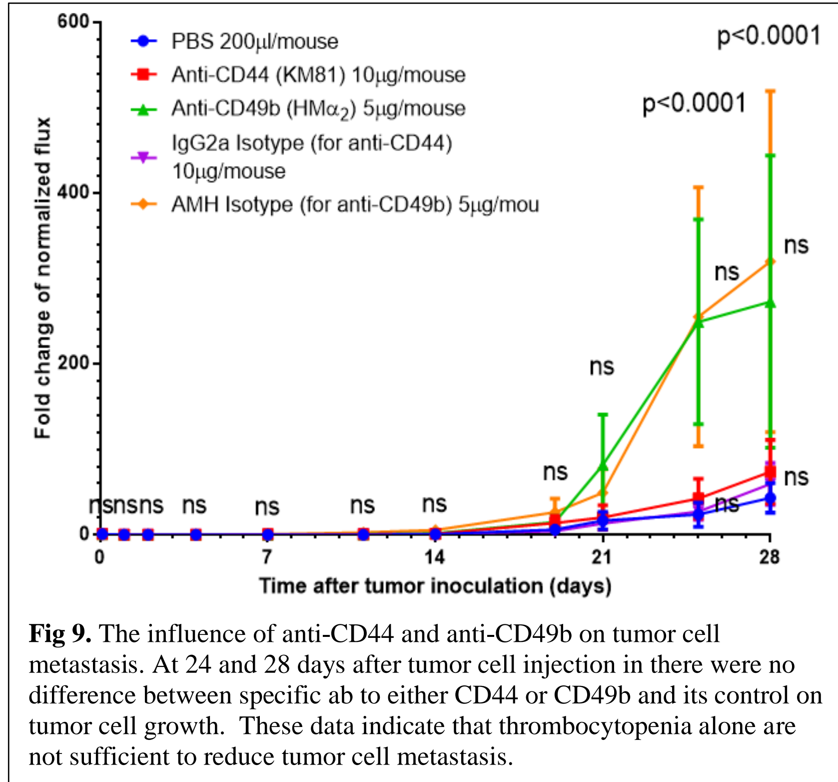

Fig 9. The influence of anti-CD44 and anti-CD49b on tumor cell metastasis. At 24 and 28 days after tumor cell injection in there were no difference between specific ab to either CD44 or CD49b and its control on tumor cell growth. These data indicate that thrombocytopenia alone are not sufficient to reduce tumor cell metastasis.

Anti-CD41 is directed to the integrin α2b, an important integrin on platelets that participates in their adhesion and aggregation to adhesive glycoproteins. It alone is a stronger target that either CD44 or CD49b that are directed to collagen and basement membrane material or directed to alpha integrin, respectively.

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, this project served as an initial career development project for Peng Zeng, MD. When he arrived in the laboratory in March 2019, he took charge of the project. The presentation in this report is the result of his efforts. This project was his PhD initial 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.

This work was presented to the Pharmacology Graduate Student seminar day in the Fall, 2019. It is under consideration for preparation for publication.

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

Not relevant. This report is a final technical report.

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 will not turn out to be high impact. After determining that platelet CD44 and CD49b have no influence on metastasis, we realized that platelet integrin $\alpha_2\beta_3$ and GPIb are the major receptors influencing platelets in tumor cell metastasis. The integrin $\alpha_2\beta_3$ when it induces thrombocytopenia at the time of tumor cell injection will lead to reduced tumor cell metastasis. However, mice deficient in integrin $\alpha_2\beta_3$ have increased tumor growth in the lung. Therefore, there appears to be a complex regulatory process involved here. We chose not to pursue this topic in further studies.

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.

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. We accomplished the goals of the original investigation. Much to our disappointment, the studies' results were not supported by our original hypotheses.

Aim II. Examine the role of IL-11/ CD49b in CTC/platelet clusters and their signaling pathway.

My role in this program in collaboration with Dr. Huiping Liu was to perform the following studies

Task 2. Are platelets activated by IL-11 to cluster with CD44+ CSCs/CTCs in vitro and in vivo? No. Platelets are not activated by IL-11; IL-11 does not influence metastasis of breast cancer cells in our model.

Task 3. Is CD44 regulated by and required for IL-11 function in promoting CSC/CTC cluster formation? IL-11 does not regulate platelet CD44. We did not exam if it regulates CD44 on our breast cancer cell line.

Task 4. Does IL-11 induce CD49b expression in platelets and BCSCs? No. IL-11 cannot influence CD49 on platelets because there is no receptor of IL-11 on platelets.

Task 5. Is CD49b important for IL-11 function in CSC/CTC clustering? These experiments were not performed.

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.

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.

As indicated in the report above, my collaborator left and institution and we were delayed almost 1 year before we could get back on track for this project.

Also, 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 were a problem in the completion of the work.

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 to date.

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

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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.

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: Direct Support:

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 Direct Support

Shire Investigator-Initiated Support (Schmaier, PI) 4/1/2018-3/31/2021 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: for 1 year. Direct Support

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: Direct Support

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: Direct Support:

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: Direct Support:

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:

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 4 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 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. Further, we learned that there is no receptor for IL-11 on platelets. CD44 is on platelets and E0771 LMC cells, but CD49b is only on platelets. Additional studies show that mice are made thrombocytopenic (85% reduction in their starting platelet counts) with 10 μg injection of a heterologous antibody to CD41 within 24 h. If E0771.LMC cells with a luciferase marker are injected (3 X 10^5 cells/ml) into these thrombocytopenic mice, there is significantly less tumor seeding and growth in the lungs at 28 days and there are reduced metastatic lesions in the lungs at 22 to 28 days. Although antibody to platelet CD44 or CD49b induce thrombocytopenia in mice, they, along with IL-11 treatment of mice, have no influence on breast cancer metastasis in this animal model. These data indicate that thrombocytopenia alone at the time of tumor cell injection is not sufficient to influences tumor cell seeding and metastasis. Reduction in tumor lung metastasis is not produced by thrombocytopenia alone, but by interference with platelet beta integrin (CD41) not CD44 or CD49b (platelet alpha integrin).