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TITLE: Defining Tumor Cell and Immune Cell Behavior in Vivo during Pulmonary Metastasis of Breast Cancer

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14. ABSTRACT Metastasis is mediated by a complex set of factors, few of which are well understood. While a number of studies over the past several decades have linked function of the immune response to various outcomes in pulmonary metastasis it is currently poorly understood as to how immune cells engage with tumor cells to modulate metastatic success. In this period we have applied an advanced method of live microscopy of mouse lungs to define the earliest hours of lung metastasis. We have identified a novel behavior of tumor cells during the earliest hours of metastasis whereby the generate and release large numbers of blebs (which we term microparticles.) Importantly, we have initial data to suggest that these microparticles are encountered and ingested by cells of the local immune response the outcome of which is still to be determined.					
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1) INTRODUCTION: Metastasis is mediated by a complex set of factors, few of which are well understood. While a number of studies over the past several decades have linked function of the immune response to various outcomes in pulmonary metastasis it is currently poorly understood as to how immune cells engage with tumor cells to modulate metastatic success. In this study we have sought to apply a novel method of microscopy to image lungs in live mice. This has allowed us for the first time to visualize the arrival of metastatic cells in the lung. Using this method we are currently evaluating the behaviors of Tumor cells themselves as well as the immune cells that interact with them in order to better understand the factors that dictate metastatic fitness in the lung.

2) KEYWORDS: *Metastasis, Intravital Imaging, Lung, Breast Cancer,*

3) ACCOMPLISHMENTS:

What were the major goals of the project?

Major Goals for Year 1:

- 1) Defining the Timeline of Cancer Cell Fate during pulmonary metastasis.
 - a) Refine and optimize method for intravital imaging of Lung Metastasis
 - b) Characterization of Tumor Cell Behavior During Pulmonary Seeding via 2-photon microscopy
 - c) Characterization of Tumor and Immune Cell Interaction in Metastasis.
- 2) Determine Requirement for VEGF Signaling During Pulmonary Metastasis

4) What was accomplished under these goals?

During the first year of this project we have made significant headway in addressing Major Goal One

A) Defining the timeline of cancer cell fate during pulmonary metastasis.

a. Refine and optimize method for intravital imaging of Lung Metastasis

At the initial time of application our lab had established a method for intravital 2-photon imaging of live mouse lungs¹ and we sought to apply this method to the study of pulmonary metastasis. Early on during these initial attempts we found the method as it had been

established to not be fully up to the task of getting high quality long-term imaging of metastatic cell seeding into the lung, while imaging was achievable, as we had previously shown, the stability of this method and overall well being of the animal was less than we had

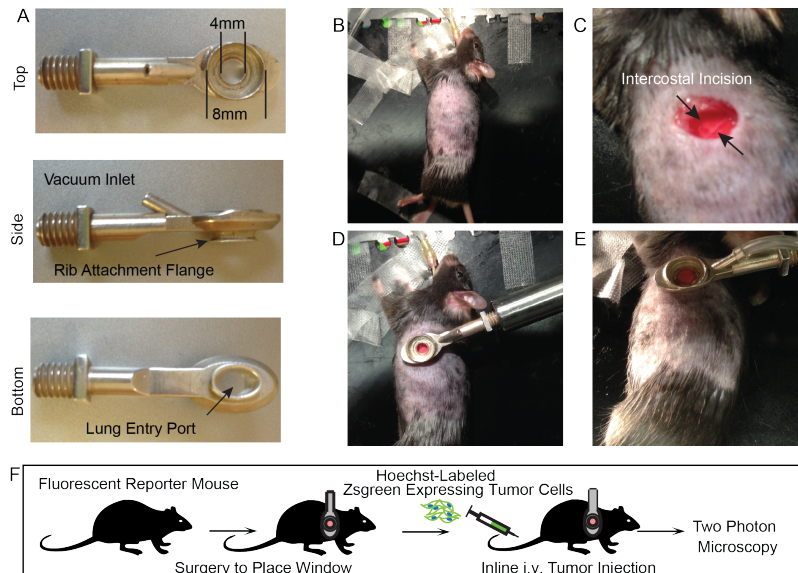


Figure 1: Intercostal Insertion Window for Lung Intravital Microscopy
A.) Top, side, and bottom views of the intercostal insertion window. Window accommodates an 8 mm coverslip and allows for visualization of 4 mm field of the left lung lobe.
B-E.) Images detailing surgical insertion of intercostal window. B.) Mouse is intubated, attached to ventilator, and placed in right, lateral decubitus position and surgical field is shaved. C.) ~6 mm incision is made immediately above ribs 4 and 5 over the anterior surface of left lung lobe. D.) Intercostal window is slipped between ribs 4 and 5 and attached to a rigid support. E.) ~20 mm Hg of vacuum suction is applied to window to secure a small portion of lung to the coverglass. F.) Schema showing approach for 2P-IVM of lung seeding by tumor cells.

anticipated. We as such returned to the development phase and dramatically redefined our approach to lung intravital imaging and have since established a novel method (**Figure 1**) utilizing an intercostal imaging window which does not require excision of ribs as was necessary in the previous incarnation of the method. Utilizing this modified approach (Lung Intravital

Microscopy, LIVM) we have now been able to achieve stable high-resolution imaging for many hours and have applied this to the study of pulmonary metastasis. Due to the increased development time for improving our method of LIVM (designing, prototyping, and establishment of the new method took approximately 5 months) our schedule slipped somewhat for our direct research goals however we have still been able to make significant headway in addressing the primary aims of **Major Goal 1**. These were to characterize the early behavioral characteristics of prospective metastatic tumor cells as they enter the lung and exist in this new environment over the first hours to days. Additionally, we sought to better characterize how these early tumors cells interact with Immune cells during these same early hours.

b. Characterization of Tumor Cell Behavior

To evaluate tumor cell behavior early during metastasis we applied our LIVM system of a model of experimental metastasis whereby tumor cells are injected intravascularly (via tail vein injection) either inline

with imaging or prior to prepping the animal for surgery. This method has revealed a unique phenomenon by which tumor cells shed large quantities of cytoplasm-containing blebs (cytoplasts/microparticles) during the earliest moments of metastasis extending out to several hours following lung entry (Figure 2A,B, and C.) Importantly, the production of these microparticles is not unique to a given tumor line as we observed the process with two distinct breast tumor lines (Mouse and Human) as well as Melanoma and even to a lesser extent

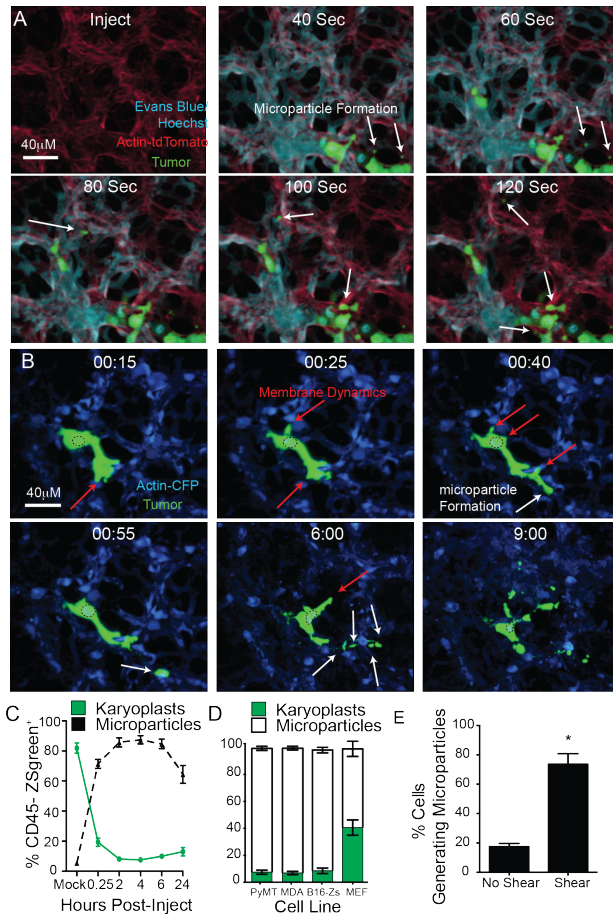


Figure 2: Intravital Imaging of The First Hours of Pulmonary Metastasis
A.) LIVM after i.v. injection of Hoechst-labeled tumor cells into mTmG mice with Evan's Blue labeling vascular flow.
B.) LIVM Hoechst-labeled tumor cells 15 minutes - 8 hours after i.v. injection into Actin-CFP recipient. A.) and B.) White arrows highlight the formation of cytoplasmic blebs (microparticles) and red arrows highlight regions of membrane activity (extension or retraction). Representative of at least 10 mice.
C.) in vivo microparticle and karyoplast frequency in lung. n=6 per group.
D.) in vivo microparticle frequency 2 hours post-injection of murine breast tumor (PyMT-B), human breast tumor (MDA-MB-231), murine melanoma B16ZsGreen, and non-transformed primary Mouse Embryonic Fibroblasts. Green: karyoplasts ; white: cytoplasts, n=3 per group from 2 experiments.
E.) Percent microparticle producing tumor cells from non-shear (slice imaging) or shear (LIVM). 15 cells per group from 3 mice. * p=0.002 by unpaired t test.

with non-transformed mouse embryonic fibroblasts suggesting this might be a general feature of cells exposed to the stress of shear in the lung environment. Further, this process was dependent on the shear forces of the lung vasculature as imaging of metastatic tumor cells in slices of viable lung tissue (in the absence of normal vascular flow) showed dramatically reduced production of cytoplasts. This finding not only reveals a unique feature of tumor cell behavior during the earliest moments of metastasis but further highlights the importance of using LIVM to study this process as it would not be observable directly by other more conventional means.

To extend our analysis of tumor behavior over time in the lung we evaluated cells at two different timepoints (2hrs and 24hrs) over 30 minute timelapses to assess how tumor cell motility/activity

changes as the cell survives for longer periods in the lung (Figure 3.) Through this analysis we found interestingly that tumor cells surviving over time in the lung become less and less active, presumably as they

become more integrated with the new environment and extravasate though this remains to be determined. An additional aspect of this

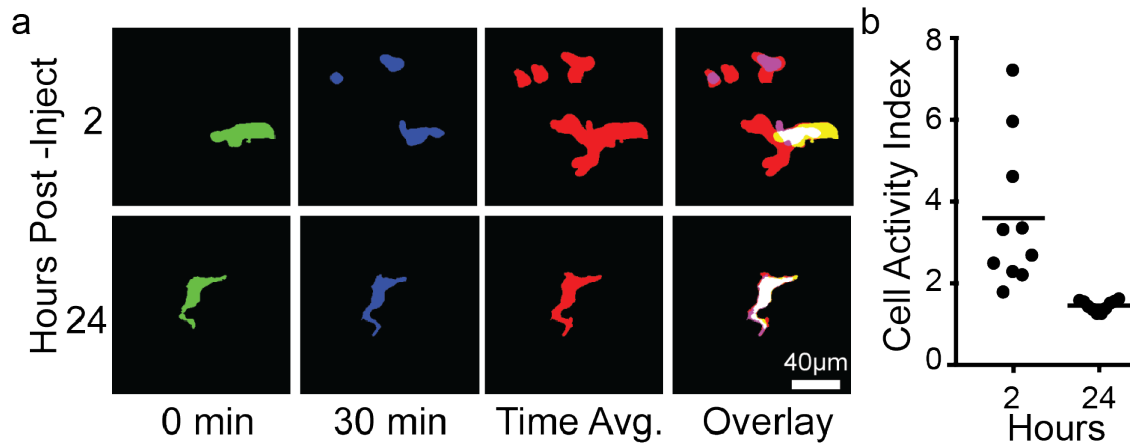


Figure 3: Assessment of Tumor Cell Activity over 24hrs

A.) Binarized maximum intensity projections of representative cells at 2 or 24 hours post-injection. Cells were time projected over a 30 minute window to assess overall cellular activity during the interval. Images show the beginning timepoint (0 min), the ending timepoint (30 min), the time projection, and the overlay. White filled space in the overlay represents the region of the cell that was stable during the analyzed interval. These data are representative from imaging performed in 3 mice.

B.) Quantification of the Cell Activity Index (defined as the area calculated from the projection of all positions over time as a ratio of the common area over all time (e.g. area of overlap)) from analysis in D (n=10 cells per group).

study as proposed had been to track proliferation and cell cycling, and survival of tumor cells over these time periods using the commercially available FUCCI and FLIVO reagents. However, though these techniques work well in confocal microscopy we unfortunately found the fluorescence levels achieved to be below the level detectable by our 2-photon setup, we are currently seeking other approaches to address these questions. However, with respect to survival we have been able to accurately determine survival through flow cytometry and observation during imaging. Using these method we have calculated a $T_{1/2}$ value for survival of metastatic cells in the lung (time from injection to ~50% of cells having died off) for the tumor cells of 6.3 hours.

c. Characterization of Tumor and Immune Cell Interaction in Metastasis.

The second major goal for the first year of these studies was to begin work on characterizing how immune cells in the lung microenvironment encounter and interact with these recently arriving tumor cells. This is a crucial question and forms the centerpiece of our ongoing study. A significant body of work over the past several decades has established the importance of a wide variety of immune

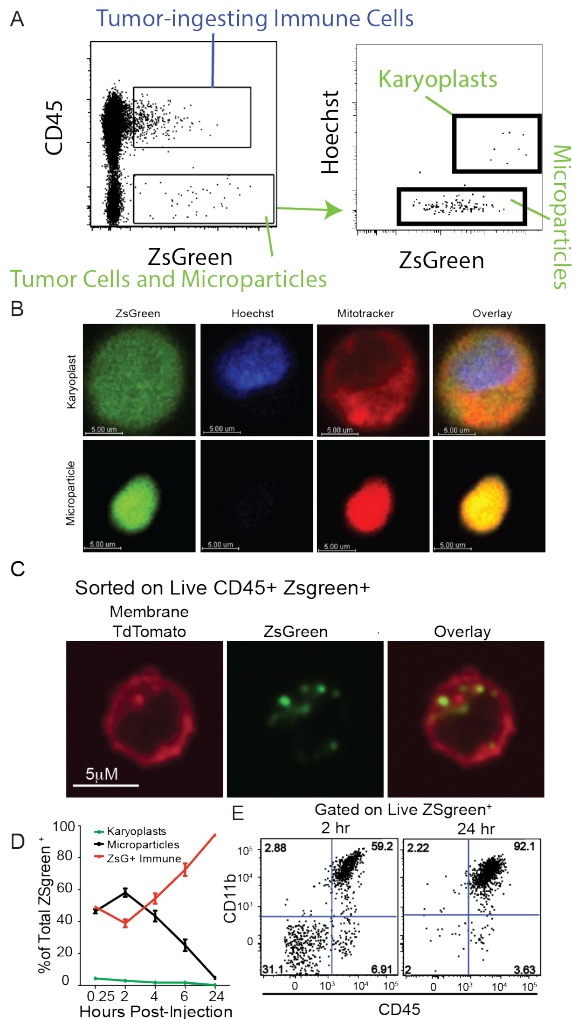


Figure 4: Loading of Lung Immune Cells by Tumor Microparticles

A) Gating strategy to distinguish nucleated tumor karyoplasts (CD45⁻, ZsGreen⁺, Hoechst⁺), anucleate tumor microparticles (CD45⁻, ZsGreen⁺, Hoechst⁻), and tumor-ingesting immune cells (CD45⁺ ZsGreen⁺) in lung.

B) Confocal imaging of sorted tumor karyoplasts and tumor microparticles and C) tumor-ingesting immune cells based on strategy proposed in A.

C.) Frequencies of microparticles, karyoplasts, and tumor-ingesting immune cells in the lung over 24 hours. n=6 per group.

D) Flow cytometry of CD11b⁺CD45⁺ cells in total ZsGreen⁺ population 2 and 24 hours post-injection.

cells in tumorigenesis and metastasis, in particular².

However, no studies have directly visualized how these cells encounter and interact with prospective metastatic cells. Due to the slight shift in timeline (to develop a more refined imaging approach) these studies are not fully realized, as of yet, however we have already made some interesting observations that we are currently seeking to better understand.

Building upon our finding that tumor cells release significant numbers of microparticles over a sustained period following entry into the lung we hypothesized that these tumor blebs may form a key element for detection and interaction by cells of the immune system. To address this question we initially developed a means to quantify not only the Tumor Cells themselves and the microparticles they produce (as in Figure 2C) but additionally immune cells that have become labeled with a fluorescent protein expressed in our tumor cells (suggesting they may have encountered and ingested the microparticles.) To achieve this we wanted to ensure the highest fidelity possible in tracking cells of this nature so we turned to a particular green fluorescent protein

(FP), ZsGreen, which has been shown to have significant photostability as well as acid stability³. Our reasoning for this was to ensure that once the Fluorescent protein enters the immune cell it will be resistant to degradation in lysosomal compartments within the cell allowing subsequent tracking of those cells. We paired use of this FP with flow cytometry on dissociated lung tissue at various timepoints to quantify Tumor cells (Karyoplasts), Microparticles, and tumor-labeled immune cells (Figure 4A). Fluorescence assisted cytometric sorting (FACS) paired with confocal microscopy was utilized to confirm the accuracy of our gating strategy and indeed as expected Tumor Karyoplasts were large nucleated ZsGreen+ cells (Figure 4B). Tumor cytoplasts were small anucleate ZsGreen+ microparticles (Figure 4B), and tumor-ingesting immune cells (TIIC) were small with ZsGreen+ vesicularized puncta held within their cytoplasm (Figure 4C). We next used this

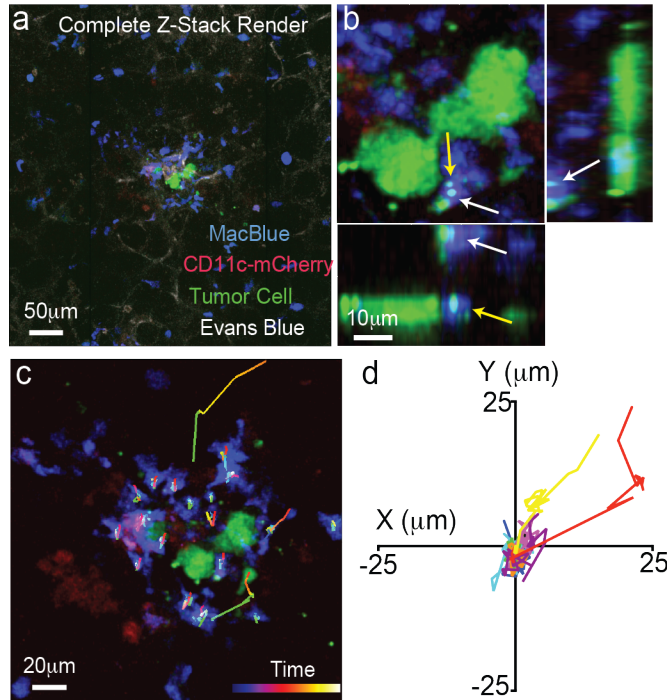


Figure 5: Tumor-Loaded Macrophages in Stable Interaction with Metastasis At 24 hours.

A.) Maximum intensity projection of LIVM of ZsGreen+ Metastatic Tumor Cells 24 hours post-injection into CD11c-mCherry x MacBlue host. Representative of 5 mice.

B.) High magnification showing tumor-loaded MacBlue+ cells in close interaction with Tumor cell at 24 hours post-injection.

C.) Representative image from time-lapse. Track overlays describe motion of tracked MacBlue+ cells over a period of ~1 hour.

D.) Overlay of 10 tracks of MacBlue+ cells from C.

method to quantify the relative frequencies of these populations over the first 24hrs following CTC entry into the lung. Our finding was quite striking that TIIC and tumor microparticles exist in a strong reciprocal relationship over this time frame, with TIIC appearing and rising in frequency at the same rate as tumor microparticles decline (Figure 4D).

At the time of this report we are just beginning our studies to elucidate the identity of the immune cells that are interacting with metastatic tumor cells during these earliest moments of metastasis. Initial work shows that at both 2 and 24hrs post injection the vast majority of cells that have become labeled with the tumor-expressed FP express the

Myeloid lineage marker CD11b (Figure 4E.) This finding suggests that it is likely that cells of the Myeloid immune system (Macrophages, Monocytes, Neutrophils, etc...) are encountering and interacting directly with incoming CTC during the initial moments of metastasis. In keeping with this idea we have successfully imaged a 24 hour metastasis in combination with a fluorescent strain that specifically marks cells of the monocyte and macrophage lineage (MACblue, expression of Cyan Fluorescent Protein driven by promoter for the Monocytic lineage *cfms*.) Consistent with our findings in Figure 4E and prior literature^{4,5}, we found MacBlue+ myeloid cells displaying clear ZsGreen+ intracellular puncta in direct and sustained interaction with tumor cells that have survived out to 24 hours in the lung. Our data shows for the first time that these interactions are truly stable with the MacBlue+ cells forming a "nest-like" structure around the burgeoning metastasis (Figure 5). Our studies over the next year will be targeted at exploring these interactions through 2-photon microscopy in addition to fully characterizing the exact identities of these tumor-ingesting immune cells.

Due to the delay associated with improving our lung imaging methodology we have not yet begun our studies focused on the molecular aspects of this model system as we are still actively characterizing the fundamental features of the tumor and immune behaviors over these early timeframes.

4) What opportunities for training and professional development has the project provided?

a) Local seminars, Lab Meetings, and Journal Clubs

- In keeping with our original training plan listed under the accepted Statement of Work I have attended regular seminar series and journal clubs held department-wide here at UCSF. Additionally, I have presented findings from this project at several Krummel Lab-specific Lab meetings as well as a Joint Lab Meeting held in combination with Dr. Zena Werb's group here at UCSF.
- This project also gave me the opportunity to present to a wider audience here at UCSF through our Tumor Immunology and Immunotherapy Journal club.
- Additionally, I also spearheaded and organize a monthly Journal Club for our Lab where we discuss papers relevant to the field of

cancer immunology and intravital microscopy.

b) Meetings and Presentations

- March 2014- During this first year of the project I attended the Keystone Meeting on Inflammation, Infection, Cancer and Immune Evolution in Cancer. I presented a poster at this meeting and was able to participate in several key conversations and discussion with faculty and peers in the Tumor Immunology field.
- May 2014 - I presented a special seminar for the Stanford University Immunology community in a special seminar about the study of lung metastasis through the use of lung intravital imaging.
- September 2014 – I was invited and presented at the Western Association of Core Directors meeting in order to disseminate our Lung Imaging methodology to other microscopy core facilities throughout universities and institutions in the country.

c) Teaching and Mentoring

- During this time I was asked to be a session lead for the UCSF Medical School Article Discussion Group. This involved leading a group of second year medical students in analyzing and discussing papers of interest to Cancer Immunotherapy.
- Additionally during this time I mentored a technician within our group who has assisted on this project. This has involved training her in animal handlings and husbandry, Cell culture, PCR, lung imaging, as well as data analysis and presentation skills.

5) How were the results disseminated to communities of interest?

During this time results were disseminated to communities of interest through presentations at a Keystone Conference on Immune Evolution in Cancer as well as at the Western Association of Core Directors Conference.

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

Years 2 and 3 of this project consist largely of furthering our studies into how the immune system functions and interacts with prospective metastatic cells in the lung as well as extending these studies into looking at molecular players in these processes with long-term goals of defining new therapeutic approaches to treating metastasis. Despite the brief setback in timeline required to improve on our lung imaging methodology we have remained largely on target with progress in these studies.

Year 2 of this project will continue to elaborate on our studies focusing characterizing how the local lung immune system responds to an interacts with incoming metastatic cells. In particular, our preliminary data suggesting that tumor microparticles might play a key role in communication between the tumor and the immune response will be an area of specific focus. We will also begin to dissect the molecular aspects of this system with an initial focus on known factors such as VEGF. Applying our refined imaging methodology to ascertain how tumor cell behavior adjusts during intervention against key elements of this system.

Year 3 of this project will largely seek to extend our findings from Years 1 and 2 temporally beyond the earliest windows of metastasis into studies focused on how these elements play out as the metastasis matures into full-blown metastatic disease. In particular, we will be focused on studies highlighting how the adaptive immune response develops and is conditioned by the metastatic environment. Our work in Year 1 to improve our lung imaging system should greatly increase the quality of our work in these future stages.

7) IMPACT:

a) What was the impact on the development of the principal discipline(s) of the project?

As part of these studies we have been able to directly observe, for the first time, the arrival of a metastatic tumor cell into the lung. While the total impact of this is as of yet unknown this is a first large step on the road to understanding the factors that allow for successful metastasis to occur. We have additionally identified a unique structure produced by tumor cells during these earliest moments of metastasis (the tumor microparticle/cytoplasm.) Importantly, we have found that the immune system appears to encounter and “eat” these microparticles. It is currently our theory that this interaction forms a platform by which tumor cells are able to communicate with their local environment and

possibly further their own successful growth in the lung. We are excited to test these ideas, hopefully to increase our understanding of metastasis in general and lead to better targeted therapy.

b) What was the impact on other disciplines?

Over the first year of this project we have made several key developments that should provide both useful methodology and data for future studies. Primarily, our work at the beginning of the year (shown in Figure 1) to improve the stability and long-term nature of our Lung Live Imaging method will have substantial impact on a variety of studies involving lung imaging. The specific accomplishment here is through use of the intracostal imaging window we have greatly improved the stability of our imaging approach – in practical terms this means that we have better ability to visualize low intensity signals as well as rapidly moving cells within the lung environment. We have already trained one of our collaborators (Dr. Mark Looney) in this new method which he is now using to study platelet production and circulation in the lung vasculature. Additionally, other individuals in our own lab are using this method to study immune cell behavior in the disease Asthma as well as T cell behavior in lung viral infections. This method will hopefully enable greater overall imaging of the lung in a variety of contexts.

c) What was the impact on technology transfer?

Nothing to Report.

d) What was the impact on society beyond science and technology?

Nothing to Report

8) CHANGES/PROBLEMS:

a) Changes in approach and reasons for change

Nothing to Report

b) Actual or anticipated problems or delays and actions or plans to resolve them

As described above, early during our initial studies we found that our primary method of intravital imaging was not sufficient for clear and accurate longterm imaging of pulmonary metastasis. In part this was do to the stability of this prep as it became clear early on that tumor cells arrive within the lung with seconds of injection and to visualize this accurately a great deal of imaging stability is required. We have tackled this initial problem with a new method of lung imaging (presented in Figure 1 above). Development of this method delayed progress on other elements

of the project by several months however we are currently on track and don't expect further delay.

d) Changes that had a significant impact on expenditures

Nothing to Report

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

f) Significant changes in use or care of human subjects

Nothing to Report/Not Applicable

g) Significant changes in use or care of vertebrate animals.

Nothing to Report

h) Significant changes in use of biohazards and/or select agents

Nothing to Report

9) PRODUCTS:

a) Publications, conference papers, and presentations

i. ***Journal publications.*** Nothing to Report

ii. ***Books or other non-periodical, one-time publications.*** Nothing to Report

iii. ***Other publications, conference papers, and presentations.*** Nothing to Report

b) Website(s) or other Internet site(s) Nothing to report

c) Technologies or techniques

Refined approach to Lung Intravital Microscopy (LIVM.) This approach will be described in an eventual publication on this work. Additionally, we have already shared this revised method with several other researchers onsite at UCSF as well as external labs with whom we'd previously trained in the former lung imaging methodology. We will freely train and or share knowledge with other groups interested in this method.

d) Inventions, patent applications, and/or licenses

Nothing to Report

e) Other Products

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

- i. Name: Mark Headley
Project Role: PI
Research Identifier (ORCID): orcid.org/0000-0002-0924-9326
Nearest Person Month Worked: 12
Contribution to Project: PI and Primary Researcher
Funding Support: DOD Award W81XWH-13-1-009 (BC120097)
- ii. Name: Alyssa Nip
Project Role: Research Technician
Research Identifier: NA
Nearest Person Month Worked: 4
Contribution to Project: Cell line generation and maintenance and animal husbandry
Funding Support: NIH

APPENDICES: NA

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