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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Leptomeningeal metastases (LM) are growths of breast cancer in the linings of the brain and spinal cord and/or in the cerebrospinal fluid (CSF). LM represent 11-20% of central nervous system (CNS) metastases. They are prevalent in younger patients. LM occur in all subclasses of breast cancer and are currently treated with intrathecal (IT) methotrexate or liposomal cytarabine or radiation therapy. Although 68% of patients initially responded to chemotherapy treatment, median overall survival was 18 weeks, resulting from LM progression. Severe complications arise from LM. There is an urgent need for research into this devastating form of breast cancer progression to identify new potential preventives and treatments. Despite an ongoing partial shutdown due to Covid19, we have made significant progress in deriving models for leptomeningeal invasion (LI).

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1. Introduction:

Leptomeningeal metastases (LM) occur in the linings of the brain and spinal column, and in the cerebrospinal fluid (CSF). They are most prevalent in breast cancer, particularly in the triple-negative and lobular subtypes. There are no effective treatments for LM, nor are there adequate mouse models with which to generate supporting preclinical data. The purpose of this grant is to make a panel of mouse models of LM disease, and to use these models to credential potential therapies.

2. Keywords: Leptomeningeal, metastasis, breast cancer, CNS, CSF

3. Accomplishments:

Initial work on this grant began in June, 2019 owing to negotations between the NCI and Geneva Foundation. Work was then halted in March, 2020, and resumed partially in August, 2020 due to the Covid-19 outbreak. Work to date exclusively addresses Aim 1.

Aim I. Establish multiple preclinical models of LM, either from extension of parenchymal brain metastases or direct colonization of the CSF.

a. Test existing brain metastasis models for extension into the CSF.
b. Develop new models of LM from direct injection into the CSF, prioritizing immune competent models.
c. For each model system, develop quantifiable endpoints. Determine the permeability of the blood-CSF barrier.
d. Molecularly profile the model systems using RNAseq to identify potential mechanistic and druggable pathways.

1. Introduction:

Leptomeningeal metastases (LM) occur in the linings of the brain and spinal column, and in the cerebrospinal fluid (CSF). They are most prevalent in breast cancer, particularly in the triple- negative and lobular subtypes. Recently several new therapeutic strategies have been tested for LM[1-3], but relatively few for breast cancer patients, generally as part of an all-solid-tumor enrollment trial[4-6]. Many are not based on good preclinical data and unsurprisingly, results are disappointing.

The purpose of this grant is to make a panel of mouse models of LM disease, and to use these models to credential potential therapies.

2. Keywords: Leptomeningeal, metastasis, breast cancer, CNS, CSF

3. Accomplishments:

Work completed from last year's report continues to address Aim 1.

Aim I. Establish multiple preclinical models of LM, either from extension of parenchymal brain metastases or direct colonization of the CSF.

a. Test existing brain metastasis models for extension into the CSF.
b. Develop new models of LM from direct injection into the CSF, prioritizing immune competent models.
c. For each model system, develop quantifiable endpoints. Determine the permeability of the blood-CSF barrier.

d. Molecularly profile the model systems using RNAseq to identify potential mechanistic and druggable pathways.

As stated in our previous report, we adopted the method to develop LM models from Dr. Adrienne Boire of MSKCC. In brief, cell lines, either brain tropic or normal, are adapted to leptomeningeal growth via direct injection into the cisterna magna (intrathecal/intracisternal injections, i.t.) of mice several times. Cells isolated from the meninges are then harvested and expanded *ex vivo* for a final round of intracardiac injection (i.c.)[7]. The purpose of the i.c. injections is to determine if the model successfully colonizes the LM space and to evaluate the level of parenchymal involvement. Using this method, Dr. Adrienne Boire, has made a single LM mouse model using MDA-MB-231 TNBC cells which we were going to use as a positive control in our lab. Unfortunately, we were not able to validate her model and, as we describe below in this report, we have had variable results using this method. We could not even validate the LM cells she sent us.

In the past year we have explored different strategies to optimize the derivation of LM models. While we had seen some increase in tumor cells in the LM space, it tended to correlate with increased metastasis everywhere, which is not tropism. We have run intracisternal and intracardiac injections of cancer cells in parallel with the objective of isolating cancer cells that are able to complete the metastatic process and colonize the leptomeningeal space (tropism) with minimal systemic involvement (specificity). We also hypothesized that tropism may be more abundant in certain animals, but not others. Thus, if we pool the LM washings from all animals the tropic tumor cells may be diluted out. This lead to a huge experiment where we cultured LM from individual mice and injected each into 5-10 animals. This too was a disappointment.

As of 09/24/2021we have completed several rounds of intracardiac injections for a triple negative (immune competent) and a HER2+ brain tropic (immune deficient) breast cancer models. We have also completed several rounds of i.t. and i.c. of these models run in parallel as stated in our protocol modification. A new lobular breast cancer line is also scheduled for early October.

The completed and scheduled worked is shown in Table 1.

Table 1. Scheduled dates of injections for each stage of model development.

			Route of injection					
	Cell line	**Subtype**	i.t.	i.c	i.t.*	i.c.*	i.t.*	i.c.*
Brain Tropic cell lines	JIMT-1-Br3	HER2+	**10/14/20**	**10/14/20**	**1/27/21**	**1/27/21**	**5/26/21**	**5/26/21**
Tumor cell lines	4T1 luc2	Triple-negative	**10/06/20**	**10/06/20**	**1/25/21**	**1/25/21**	**5/18/21 and 5/20/21**	**5/18/21 and 5/20/21**
	SUM44PE	Lobular			10/06/21	10/06/21		

Note: i.t. = intrathecal, i.c. = intracardiac, * Modified methods.
Bold: completed

All lines are luciferase-labelled which allow us to sequentially monitor disease burden using bioluminescence imaging (BLI).

Data for **JIMT-1-Br3 Luc/GFP:**

Round 4th of i.c. and i.t. injections from pooled cells isolated from 3rd round of i.t. injections follow below:

Cell line	Human/ Mouse	# of animals	# of cells	Volume	ROUTE	Implantation date
JIMT-1-Br3 Luc/GFP (ROUND 4)	Human	10	1.75x10^5	10uL	Intrathecal	10/14/20
JIMT-1-Br3 Luc/GFP (ROUND 4)	Human	10	1.75x10^5	100uL	Intracardiac	10/14/20

BLI imaging of these mice show the expected growth in the ventricular region of the brain via i.t. injections (Fig. 1 and Fig. 2). This is not evident for the i.c. injections. The signal in the i.c. injected mice seem to occupy non-CNS organs compared to the i.t. imaged mice where the signal runs along the spinal column. Furthermore, we were not able to isolate LM cells from the i.c. injected mice suggesting that these cells were not able to reach the leptomeningeal space.

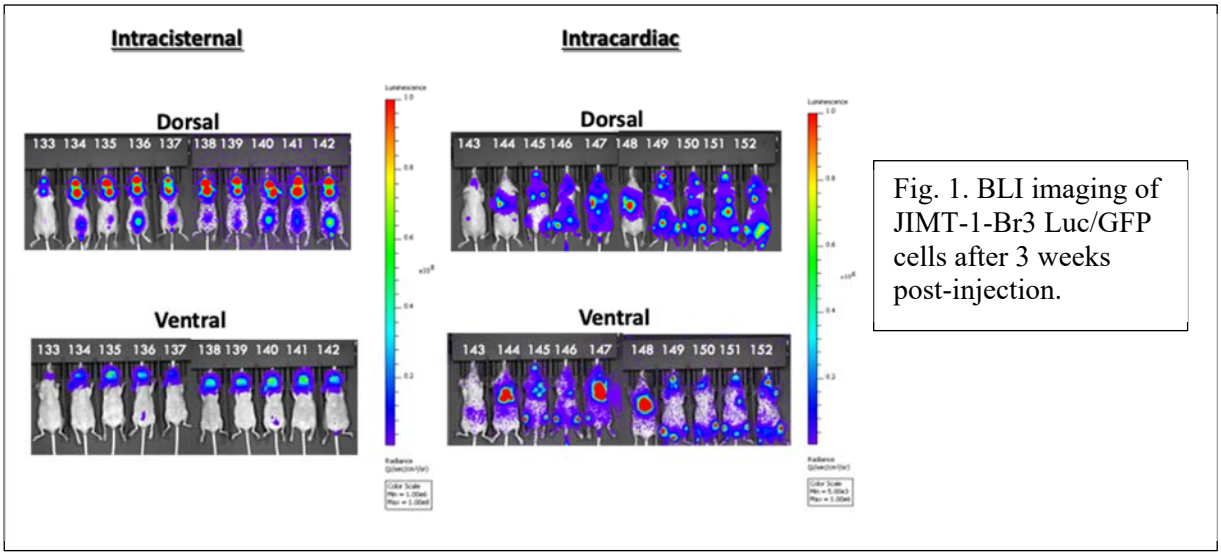


Fig. 1. BLI imaging of JIMT-1-Br3 Luc/GFP cells after 3 weeks post-injection.

Histopathology of these two groups confirmed the expected outcome from both routes of injection. Coronal sections of brains were examined with routine H&E for each mouse. Intrathecally injected mice produced predominantly meningeal masses (Fig. 3A). In contrast, i.c. injected mice produced predominantly

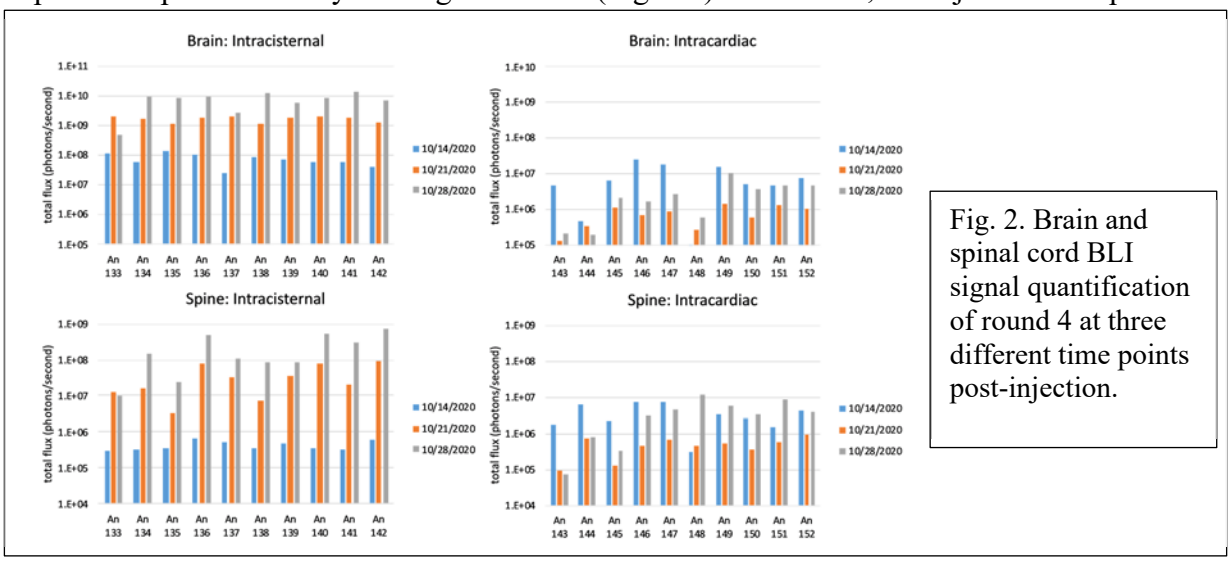
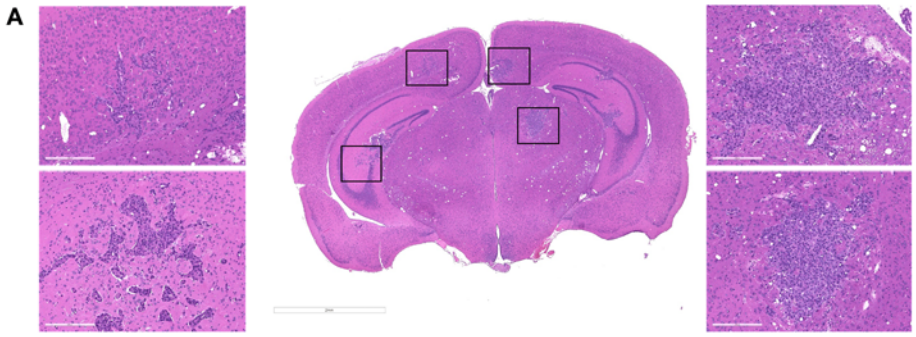


Fig. 2. Brain and spinal cord BLI signal quantification of round 4 at three different time points post-injection.

neuroparenchymal masses (Fig 3B). Different areas of the brain and spinal cord were analyzed for the presence of neoplastic cells (Table 2). By the i.t. route 5/8 mice were positive for LM growth. However, each also had accompanying parenchymal metastases, not surprising since it was started from a brain-tropic line.



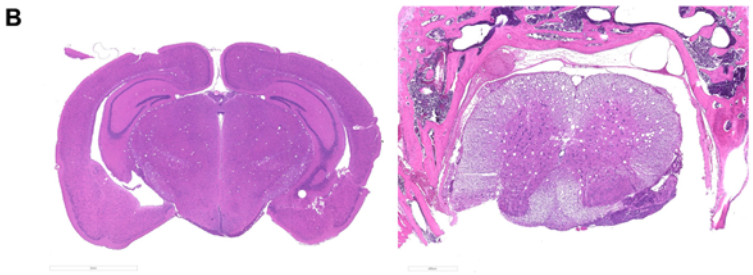


Fig. 3. Sample H&E images of (A) i.c. injected mice and (B) i.t. injected mice showing a different pattern of neoplastic cells distribution.

Table 2. Histopathology summary.

Group	Cortex	Striatum	Thalamus	Hypothalamus	Hippocampus	Medulla	Cerebellum	Meninges	Spinal cord, neuroparenchyma	Spinal cord, meninges	Anatomic locations involved
intracisternal				y		y	y	y			4/10
intracisternal		y		y		y	y	y		y	6/10
intracisternal		y		y		y	y	y		y	6/10
intracisternal		y		y		y	y	y		y	6/10
intracisternal		y				y	y	y			6/10
intracisternal				y		y	y	y		y	6/10
intracisternal		y	y	y	y	y	y	y			7/10
intracisternal		y		y		y	y	y		y	6/10
intracardiac	y	y	y	y	y	y	y		y		8/10
intracardiac	y	y	y		y	y	y		y		7/10
intracardiac	y	y	y	y	y	y	y		y		8/10
intracardiac	y	y	y	y		y	y				6/10
intracardiac	y	y	y	y	y		y				6/10

In summary, the i.c. injection route did not produce a LM line suggesting that the model is not able to complete the metastatic process and colonize the LM space. Based on these results, we decided to modify the protocol with the objective of isolating cancer cells from the meningeal space via direct i.c. injections, rather than several rounds of i.t. injections. After two rounds, we were able to isolate LM clones from two mice out of ten (Fig. 4). Previously, we would have pooled these isolated clones and injected them into ten mice for another round of selection. This time we decided to keep them separate since we are looking to find a clone that selectively colonizes the meninges (tropism) with decrease systemic involvement (specificity). These rounds are soon to be scheduled.

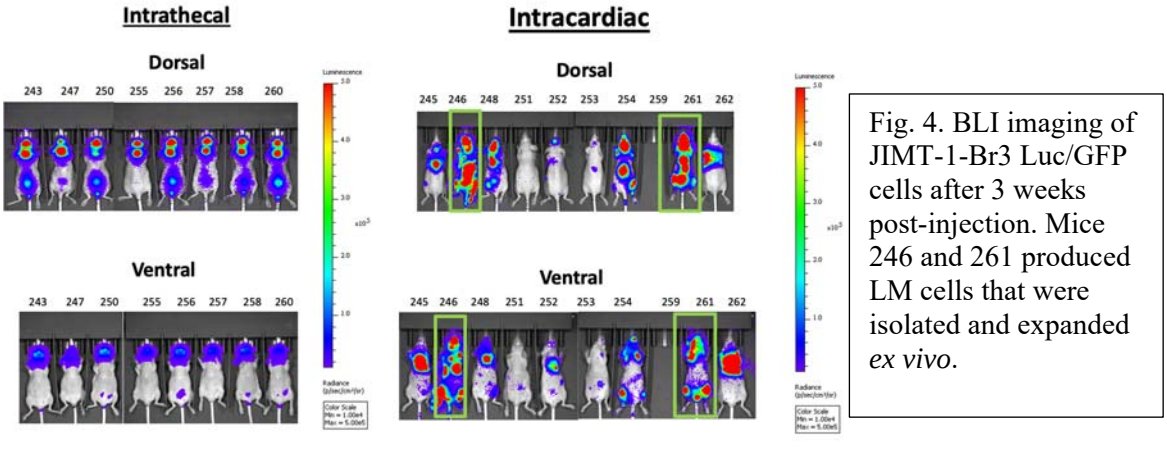


Fig. 4. BLI imaging of JIMT-1-Br3 Luc/GFP cells after 3 weeks post-injection. Mice 246 and 261 produced LM cells that were isolated and expanded *ex vivo*.

Data for **4T1 luc2:**

Round 4th of i.c. and i.t. injections from pooled cells isolated from 3rd round of i.t. injections follow below:

Cell line	**Human/ Mouse**	**# of animals**	**# of cells**	**Volume**	**ROUTE**	**Implantation date**
4T1-Luc2 (ROUND 4)	Mouse	10	5x10^4	10uL	Intrathecal	10/06/20
4T1-Luc2 (ROUND 4)	Mouse	10	5x10^4	100uL	Intracardiac	10/06/20

BLI imaging of these mice showed the expected growth in the ventricular region of the brain via i.t. injections. The signal in the i.c. injected mice was not clear compared to the i.t. imaged mice. However, we were able to isolate LM cells from one mouse out of ten from the i.c. injected mice. We expanded this clone *ex vivo* and re-injected into another ten mice for another round of selection (Fig. 5 and Fig.6). BLI images of the i.t. injected mice showed the expected signal as previously observed. The signal in the i.c. injected mice seemed to occupy non-CNS organs. This round of selection produced LM cells from five mice out of ten in the i.c. injected mice. These results suggest that we are enriching for clones that can complete the metastatic process and grow in the meningeal space, though tropism remains a complication.

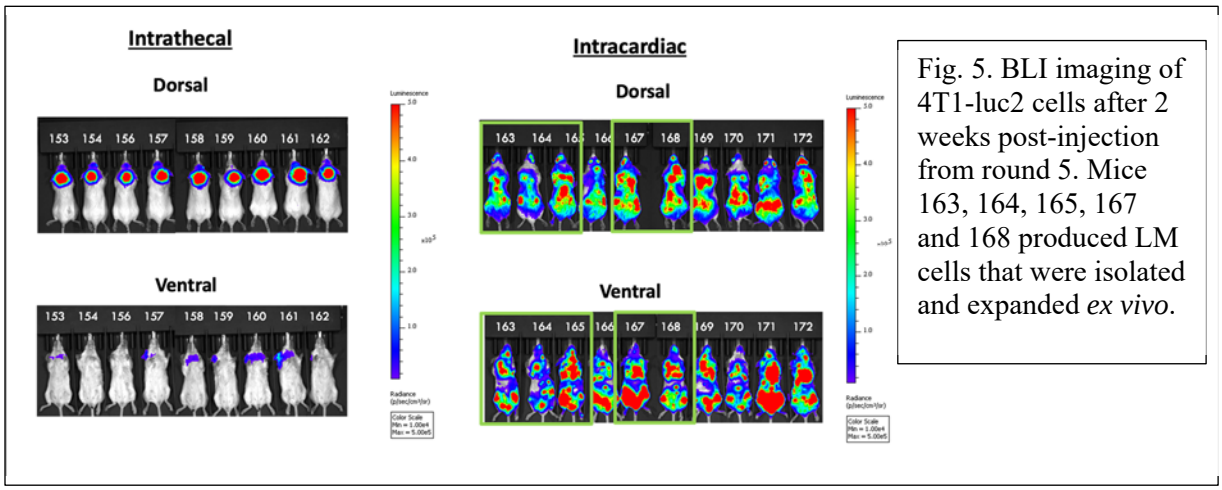


Fig. 5. BLI imaging of 4T1-luc2 cells after 2 weeks post-injection from round 5. Mice 163, 164, 165, 167 and 168 produced LM cells that were isolated and expanded *ex vivo*.

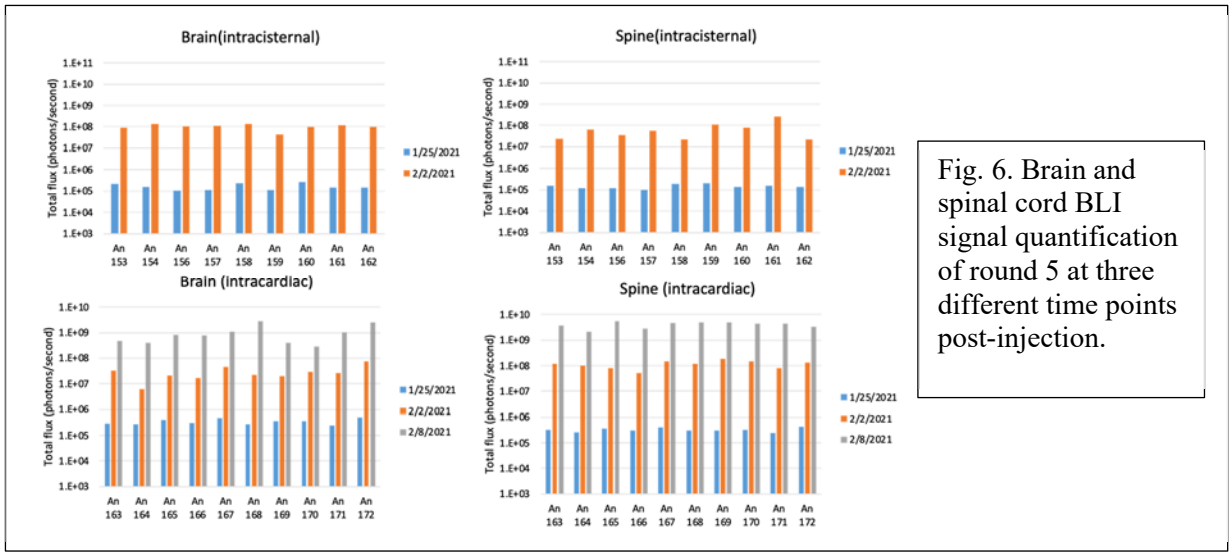


Fig. 6. Brain and spinal cord BLI signal quantification of round 5 at three different time points post-injection.

As previously stated, we not only look for tropism but also specificity. For this reason, we again decided to expand these cells *ex vivo* from each individual mouse, treating them as separate clones for another round of selection (Round 3).

Round 3 of i.c. injections from clones isolated from 2nd round of i.c. injections follow below:

Cell line from mouse # above	**Group**	**# of animals**	**# of cells**	**Volume**	**ROUTE**	**Implantation date**
4T1-Luc2 clone 163 (ROUND 3)	1	10	5x10^4	100uL	Intracardiac	5/18/21
4T1-Luc2 clone 164 (ROUND 3)	2	10	5x10^4	100uL	Intracardiac	5/18/21
4T1-Luc2 clone 165 (ROUND 3)	3	10	5x10^4	100uL	Intracardiac	5/18/21
4T1-Luc2 clone 167 (ROUND 3)	4	10	5x10^4	100uL	Intracardiac	5/20/21
4T1-Luc2 clone 168 (ROUND 3)	5	10	5x10^4	100uL	Intracardiac	5/20/21

Terminal BLI imaging of these mice show that the signal occupies non-CNS organs as previously shown (Fig. 7). Isolation of clones from the meninges for each group showed a clear enrichment in LM cells. These cells were isolated and expanded *ex vivo*. Based on the BLI images we did not observe a distinct LM specificity from any clone. This could be the result of keeping the model to long allowing these highly aggressive lines to metastasize systemically. For that reason, we are modifying the frequency of BLI imaging to being able to capture LM involvement at earlier stages. A pilot experiment for this mode of imaging is scheduled to start 9/28/2021.

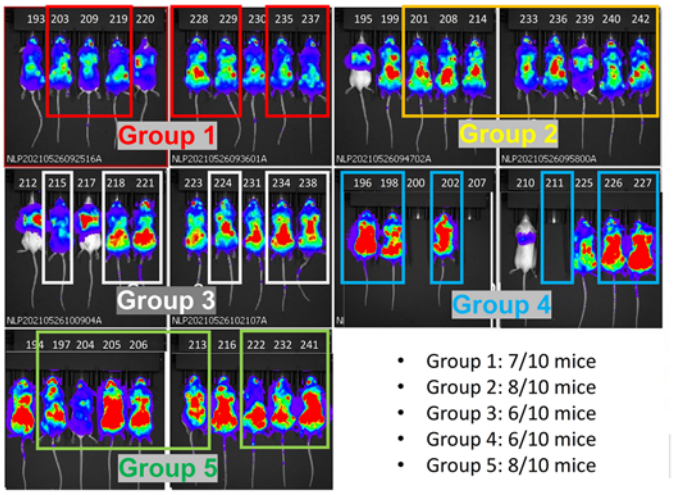


Fig. 7. BLI imaging of 4T1-luc2 cells of i.c. injected mice after 2 weeks post-injection from round 3. Number of mice for each group that produced LM cells are highlighted.

Overall summary from the JIMT-1-BR and the 4T1 models:

We are developing LM lines that can colonize the meningeal space. We find that isolating cells from the meninges of i.c. injected mice produce cells with an increased capacity to colonize the leptomeninges (metastatic assay) in terms of percent positive mice. However, tropism remains a problem and would complicate any preclinical experiment.

New approach:

What has been clear in each of these experiments is that, if we inject tumor cells into the ventricle, they spread down the spinal column consistently. This is not metastasis, its leptomeningeal invasion (LI). We hypothesize that LI represents a valid endpoint for further translational research. As such, we now have achieved this in both model systems.

I spoke with Dr. Boire in a recent online meeting and asked her if she considers LI an acceptable endpoint, and she concurred. What we will now do is optimize each model to have intense imaging, so that we can visualize LI. These experiments are planned in the coming year. It is notable that, in their most recent paper, invasion was used as an endpoint as well[8] . In addition, a similar model was described for melanoma[9] and leukemia[10].

These LI cell lines, and the starting cell lines are scheduled to be profiled by RNAseq in the coming months to identify potential mechanistic and druggable pathways.

SUM44PE cell line model.

A new model is scheduled for 10/06/21. This model is SUM44PE which is a lobular breast cancer subtype. Recent studies have shown that lobular breast cancer accounts for about 35% of all LM cases with only 7% developing intracranial parenchymal lesions suggesting a higher propensity for lobular breast cancer to spread to the leptomeninges.

This cell line grows extraordinarily slowly *in vitro*. We plan to allow the models to continue for months to allow LM or LI growth. Our survey of other lobular lines reveals that they all grow slowly *in vitro*, so this may be a class effect.

For Aim 2, profiling of human LM specimens, we have amended the tissue collection protocol from the

Women's Malignancies Branch, CCR, NCI and it is submitted for IRB review.

For Aim 3, we will not work with the LI models to begin immune profiling.

1 Lu, Z. Q. *et al.* Osimertinib combined with bevacizumab for leptomeningeal metastasis from EGFR-mutation non-small cell lung cancer: A phase II single-arm prospective clinical trial. *Thoracic Cancer* **12**, 172-180, doi:10.1111/1759-7714.13738 (2021).

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3 Nosaki, K. *et al.* Erlotinib forNon-SmallCell Lung Cancer with Leptomeningeal Metastases: A PhaseIIStudy (LOGIK1101). *Oncologist* **25**, E1869-E1878, doi:10.1634/theoncologist.2020-0640 (2020).

4 Pan, Z. Y. *et al.* Intrathecal pemetrexed combined with involved-field radiotherapy as a first-line intra-CSF therapy for leptomeningeal metastases from solid tumors: a phase I/II study. *Therapeutic Advances in Medical Oncology* **12**, doi:10.1177/1758835920937953 (2020).

5 Jaeckle, K. A. *et al.* Intra-CSF topotecan in treatment of breast cancer patients with leptomeningeal metastases. *Cancer Medicine* **9**, 7935-7942, doi:10.1002/cam4.3422 (2020).

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7 Boire, A. *et al.* Complement Component 3 Adapts the Cerebrospinal Fluid for Leptomeningeal Metastasis. *Cell* **168**, 1101-+, doi:10.1016/j.cell.2017.02.025 (2017).

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10 Brandsma, D. *et al.* Constitutive integrin activation on tumor cells contributes to progression of leptomeningeal metastases. *Neuro-Oncology* **8**, 127-136, doi:10.1215/15228517-2005-013 (2006).

4. Impact.

To date, we are unable to confirm that the initially published LM metastatic line is actually LM-tropic. This underscores the importance of obtaining additional model systems, and perhaps of identifying a new way of making LM-tropic lines from what has been published.

Despite intensive effort using several model systems, we have been unable to obtain LM-tropism (without greater systemic metastasis) to date. However, we consistently see leptomeningeal invasion (LI) from ventricular injection of tumor cells in two models and intend to develop this as our workhorse model. To do so, we will perform more intensive imaging and have our collaborator, Dr. Lyle, obtain spinal pathology readouts. We are thus ready to start on the additional aims, as well as expand our inventory of LI models.

5. Changes/Problems.

Potential changes and problems are described in the section above.

6. Products.

We are finalizing two LI model systems.

7. Participants & Other Collaborating Organizations.

Dr. Vanesa Sylvestri, a postdoctoral fellow, was employed by this grant. I have moved her to my lab funding in order to make the DOD funds available for mouse work. Dr. Tiffany Lyle, Purdue University, has completed multiple rounds of CNS pathology.

8. Special Reporting Requirements.

None

9. Appendices

None.