KPCRTF State Funded Projects Reporting Template

University of Kentucky
Macrophage derived Engineered Vesicles for Preventing Metastases in Pediatric Osteosarcoma
Program Director: Jill Kolesar

Reporting Period: April 1, 2021 through June 30, 2021

Below please provide a brief summary of the status of the Project listed as well as for each Objective listed below. Include any barriers, how and if they were overcome, and successes achieved.

Project Status

Deliverable	Due Date	Completion Date	Barriers	Notes
1a) Biosafety Protocol Approval	12.31.20	7.22.20	None	
1b) Animal Protocol Approval	12.31.20	7.13.20	None	
2) Recruit and train the graduate students and staff outlined in this proposal	12.31.20	9.30.20	None	Postdoctoral fellow Alexandra Nail, PhD has been recruited to the project following her return from maternity leave on 7.20.20. Specific trainings completed: isolation of human monocytes from PBMCs 8.14.20; generation of MEVs 9.20; intratibial injections in mice 9.20
3) Inhibit SLCO2B1 and SLC22A4 with chemical inhibitors and/or siRNA to assess the influence of SCLO2B1 inhibition on macrophage polarization	12.31.20	12.31.20		We have used siRNA to inhibit SCLO2B1 and found that it did not re-polarize macrophages. Future experiments will focus on validating the role of the surface proteins identified here in macrophage polarization.
4) Screen for the specific proteins that are present on the surface of MEVs via microarray screening for 247 unique membrane proteins	12.31.20	12.31.20	None	We have screened approximately 400 surface proteins on M1EVs and M2 macrophages.
5) Quantify receptors on the vesicle surface via quantitative flow cytometric analysis using antibody-phycoerythrin conjugates and Quantibrite phycoerythrin calibration beads	12.31.20			Preliminary experiments determined that vesicles are too small to detect using quantitative flow cytometric analysis. Because of this barrier, we are now using fluorescence spectroscopy as an alternative approach.

6) Inhibit identified membrane receptors through siRNA based gene silencing to determine their role in cell targeting and uptake	12.31.20			This deliverable was delayed as it is dependent on other objectives in the grant. Screening for membrane receptors is now complete; therefore, we have started experiments with transfection of macrophages.
7) Conduct monthly meetings with Leadership team to discuss progress and problem-solve any potential barriers	12.31.20	Standing monthly meeting established and held 7.9.20, 8.5.20, 9.2.20, 10.7.20, 11.4.20, 12.2.2020	None	
Identify key molecular signaling networks responsible for MEV driven reprogramming of macrophages to a proinflammatory phenotype by computational modeling	6.30.2021			We've identified some pathways and mechanisms for reprogramming macrophages. The identification of further pathways will be dependent on experimental validation.
Test model predictions on the regulation of protein expression and protein activity such as the phosphorylation of P38	6.30.2021		Transfection reagents/methodology required optimization due to unacceptable cellular toxicity. This issue has now been overcome and model validation is underway.	
Use a gene silencing approach to knock-down expression of specific components predicted from the modeling to differentially regulate M1-like versus M2-like phenotypes	6.30.2021			
Perform qPCR on target macrophages to identify changes in gene expression levels due to exposure to the P-MEVs	6.30.2021	5.11.2021	None	Reproducible increased expression of CXCL10 and CXCL8 has been demonstrated in human M2 macrophages following exposure to P-MEVs
Generate KRIB and 143B osteosarcoma models	6.30.2021	6.22.2021	Intratibial engraftment of tumor xenografts has proven technically challenging. We have	Luciferase-labeled HOS and 143B osteosarcoma cell lines have been developed. Subcutaneous xenografts have

			decided to proceed with subcutaneous xenografts.	been successfully generated in nude mice.
Treat four cohorts (saline control, cisplatin alone, MEVs alone and cisplatin + MEVs) of mice to assess efficacy and adverse effects	6.30.2021			This deliverable has been delayed due to difficulties establishing osteosarcoma xenografts. These challenges have recently been overcome and we plan to begin these experiments in the next reporting period.
Conduct monthly meetings with Leadership team to discuss progress and problem-solve any potential barriers	6.30.2021	Standing monthly meeting held 1.6.2021, 2.11.2021, 3.11.2021, 4.8.2021, 5.13.2021, 6.10.2021	None	

Objective 1: Determine the mechanism of MEVs re-polarization of macrophages.

a. Assess the role of SLCO2b1 in macrophage polarization.

We have completed experiments using siRNA to assess the role of SLCO2b1 in macrophage polarization. We have confirmed transfection efficiency using immunofluorescence and assayed for macrophage repolarization using an ELISA assay. Our experiments revealed significant reductions in Slco2b1 mRNA for M0 and M2 macrophages; however, we did not observe a corresponding increase in proinflammatory cytokines. Therefore, we have decided to take a different approach for this objective and focus on validating the role of the top candidates identified in objective 1b and 2a in macrophage polarization.

Major conclusions

- Experiments to inhibit SLCO2b1 with siRNA have been completed
- Slco2b1 knockdown did not shift M0 or M2 macrophages to an M1 phenotype

b. Understand surface proteins responsible for MEV targeting.

We have made significant progress toward the identification of proteins on the surface of macrophage engineered vesicles (MEVs). We used a combination of western blot analysis, protein antibody arrays, and sandwich Enzyme-Linked Immunosorbent Assay (ELISA) to screen for proteins common to macrophage activation, exosomal markers, and membrane-bound proteins. We have screened for approximately 400 surface proteins, comparing those present on M1EVs to those present on our target tumor associated macrophages (M2 macrophages). Approximately 20 proteins have been identified based on high levels of expression on M1EVs, differential expression on M1EVs versus M2 macrophages, and by comparing ligand-receptor matches between proteins on M1EVs and M2 macrophages (see Figure 1, Figure 2, and Appendix). The candidates identified to this point consist of proteins previously identified as being

involved in macrophage polarization (e.g., Ccl5, Ccl2, Cxcl9, Hdac1) as well as those that have not been connected to a specific phenotype or change in polarization (e.g., cyclin E1).

In order to better understand the role of surface proteins in MEVs targeting and repolarization, we tested the ability of M1EVs without surface proteins to target and repolarize M2 macrophages. Removal of surface proteins on MEVs with proteinase K digestion results in decreased uptake of MEVs by M2 macrophages; however, MEVs uptake was still observed (Appendix). Importantly, M1EVs without surface proteins (PKT-MEVs) were unable to repolarize M2 macrophages towards the M1 phenotype (Figure 3). Protein analysis by western blot of proteinase K treated MEVs showed a decrease in CD54, CD63, and the ER marker Calnexin (Appendix). This data demonstrates that surface proteins are necessary for MEV driven macrophage repolarization.

We also performed experiments comparing plasma membrane (PM)-derived MEVs to endoplasmic reticulum (ER)-derived MEVs. While both ER-MEVs and PM-MEVs are capable of repolarizing M2 macrophages, ER-MEVs are more efficient at repolarization (Figure 4).

In future experiments, we will further refine the candidate list with additional data analysis. Will validate the candidates involvement in modifying macrophage phenotype by first quantitatively measuring the expression level using flow cytometry and then by changing expression levels and observing the effect on macrophage repolarization. We also plan to test human peripheral blood mononuclear cell-derived macrophages using the human antibody arrays.

Figure 1: Proteins present on M1EVs identified by chemokine array.

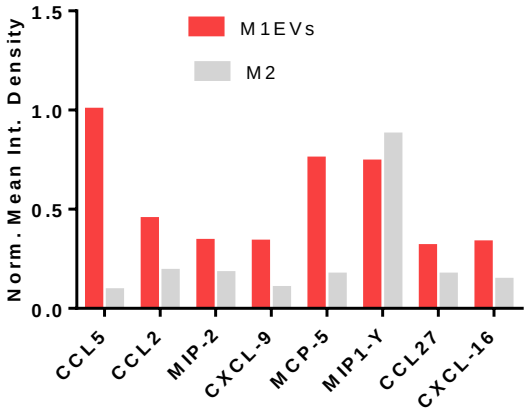


Figure 2: Macrophage associated proteins present on M1EVs identified by cell signaling array.

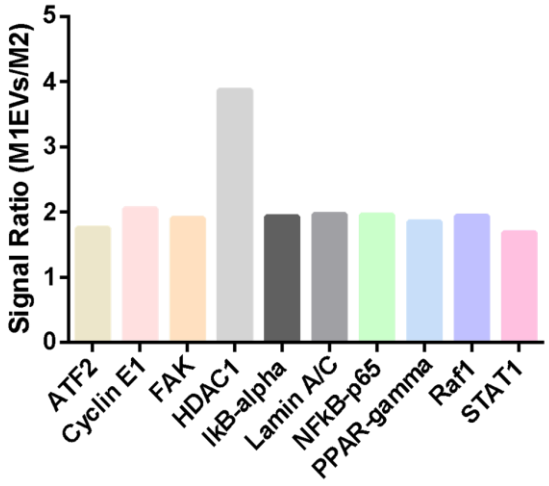


Figure 3: Elimination of surface proteins reduces the capability of MEVs to repolarize M2 macrophages.

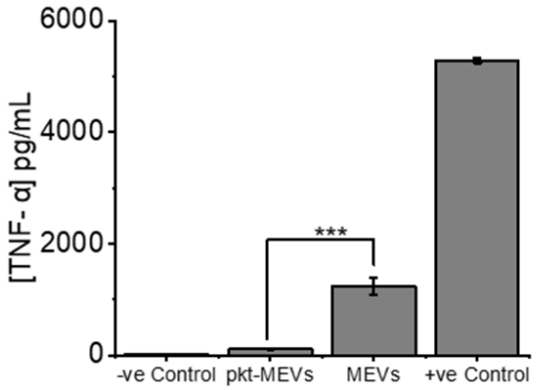
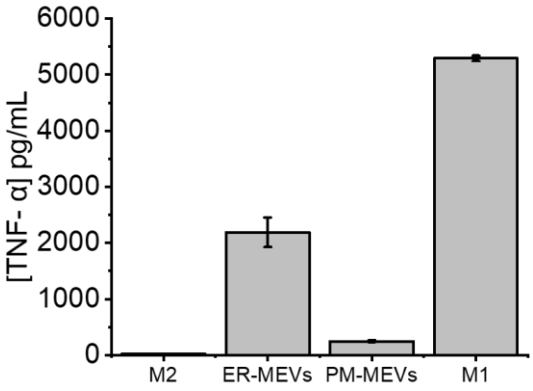


Figure 4: ER-derived MEVs are more efficient at repolarizing M2 macrophages compared to PM-derived MEVs.



Major Conclusions

- Screening for approximately 400 surface proteins on the surface of M1EVs has been completed
- Approximately 20 candidate surface proteins have been identified, including both novel and previously reported macrophage polarization proteins
- Surface proteins are required for repolarization of M2 macrophages by M1EVs
- MEVs derived from endoplasmic reticulum are more effective at repolarizing M2 macrophages than plasma membrane-derived MEVs

Objective 2: Program MEVs for enhanced macrophage polarization.

a. Identify key molecular signaling networks responsible for MEV driven reprogramming of macrophages to a proinflammatory phenotype.

The signaling networks that govern macrophage responses to external stimuli are extraordinarily complex. The complexity arises from the breadth of plasma membrane receptors that activate or inhibit signaling pathways within the cell, convergence and divergence of signals through signaling hubs such as NF-KB, and negative or positive feedback pathways that tune cellular responses. Therefore, it is advantageous to use computational techniques to exhaustively search candidate pathways and rank them by their ability to drive M1 polarization upon the activation of membrane receptors.

Our lab opted for a graph theoretic approach to query macrophage signaling pathways. The signaling networks comprise nodes representing intracellular or plasma membrane proteins, while edges linking those proteins symbolize protein/protein interactions or communication via diffusible substrate (see middle panel Figure 5). The advantage of this representation is that there are well-established algorithms for identifying optimal pathways that link two arbitrary nodes, such as a path that links receptor activation to a cytokine response typical of M1 macrophages.

Our approach is summarized in the upper right corner of Figure 5. The key steps to the approach are 1) the curation of signaling networks from databases and 2) pathway searching via network analysis algorithms. For 1), we manually collect candidate pathways implicated in macrophages from databases that include the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Wikipathways. Cytoscape is used to merge pathways into a broad network of protein/protein interactions. The resulting network topology is exported for analyses using Networkx. Networkx is a comprehensive python library for performing routine graph analyses, including the identification of 'minimum first paths' that link a given receptor to an M1-associated cytokine gene product. We additionally incorporate RNAseq and immunohistochemistry data collected by the Richards and Kolesar labs in order to align the curated network topologies with the macrophage and macrophage-engineered vesicles they are developing. Altogether, 1) and 2) yields sets of pathways and the receptors that activate them in a rank-ordered list.
Preliminary efforts with this approach thus far has yielded several actionable pathways (Table 1). The highest ranked pathways include the TNFa and interleukin 1 receptors, which activate pathways well-known to polarize macrophages to pro-inflammatory M1 states. Also identified were pathways associated with Cluster of Differentiation 81 (CD81), a member of the tetraspanin family, and C-C Motif Chemokine Receptor 3 (CCR3), a chemokine receptor. CD81 is expressed in microglia following traumatic brain injury and is implicated in the activation of these brain-resident macrophages [1]. Activation of CCR3 by the CCL5 and CCL24 chemokines induces macrophage chemotaxis, which is a hallmark of the M1 state [2]. RNAseq data collected by the Kolesar lab confirmed increased expression of CCL5 mRNA transcripts in M2 cells treated with M1-derived vesicles (logFC=2.4, where positive values indicate upregulation relative to M2). Western blots for CD81 performed by the Richards lab confirmed expression of CD81 in M1 macrophages and M1-derived vesicles, and non-expression in M2 macrophages. CCR1 and CCR5 were also identified as receptors involved in M1 polarization. CCR1 is expressed in M2 TAMs but shows much higher expression in M1 macrophages, suggesting that overexpression of CCR1 ligands in M1 MEVs may promote repolarization of M2 TAMs. Pathway analysis has suggested that a combinatorial approach might be used to maximize M2 repolarization, such as promoting M1

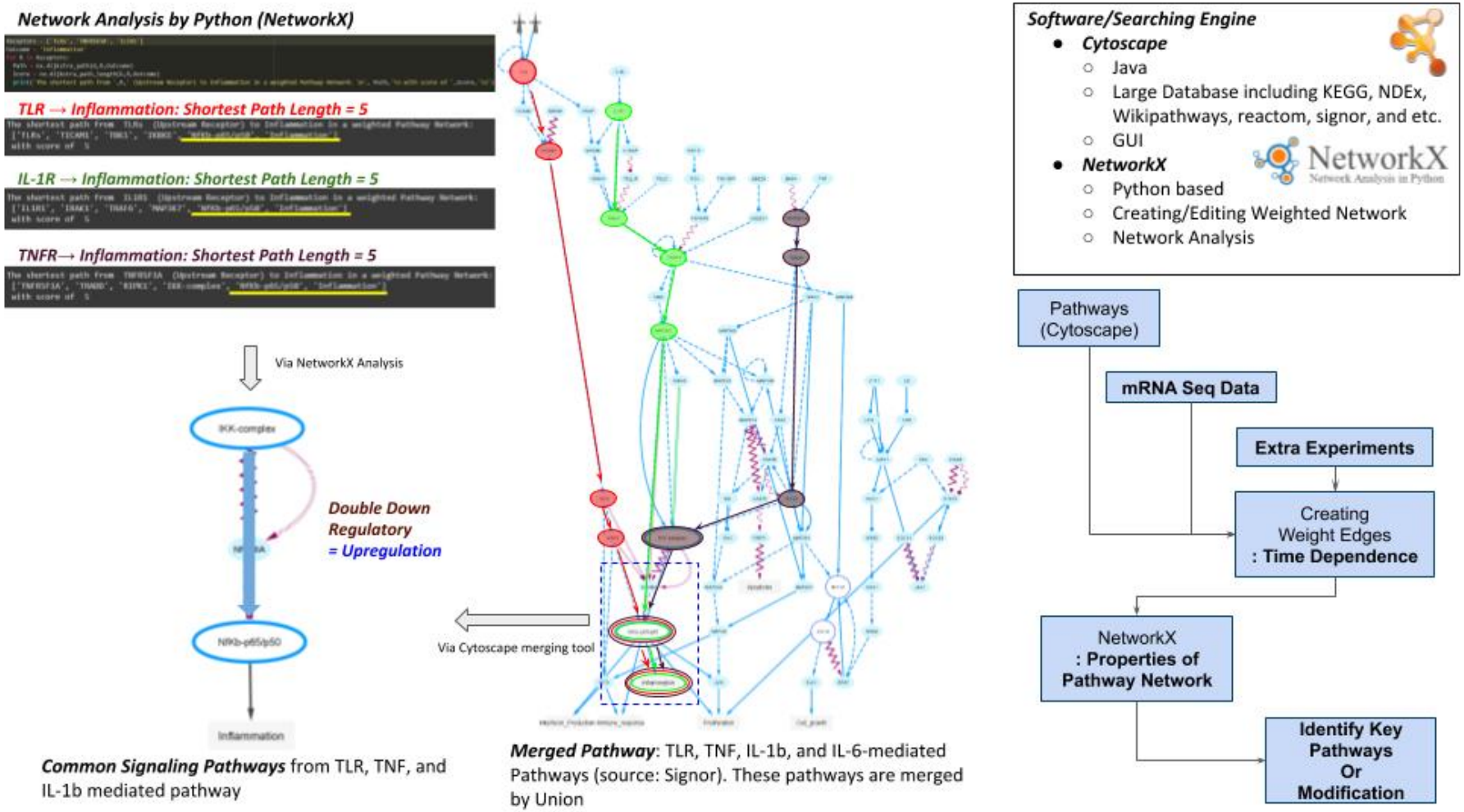
polarization by CCL5 overexpression while also promoting transcription of negative feedback regulators of M2 polarizing pathways.

We have several goals for the upcoming evaluation period. We continue to expand the network to identify additional targets. We have begun this process by automating algorithms to pull protein-protein interaction data from the STRING database. We are also revising algorithms to incorporate combinations of M1-promoting and M2-suppressing interactions, along with negative/positive feedback loops, instead of focusing solely on M1-activating (non-inhibitory) pathways. In our future work, we will consider secondary messengers that might be involved in inflammatory responses. We also plan to use monoclonal antibody data for phosphorylated enzymes that can be used as a sign of activation of different pathways. Ligands for candidate receptors will be evaluated *in vitro* for polarization potential and results will be used to further refine the network.

We are further exploring other methods for identifying proteins that repolarize M2 tumor-associated macrophages (TAMs) to M1 TAMs and connecting identified repolarizing proteins to a membrane protein through a signaling pathway. Tools and analyses methods being investigated for these goals include automated literature searches and protein-protein interaction predictions (PPIs). Automated literature searches are being investigated to predict proteins responsible for repolarization. The idea is to identify terms relevant to repolarization and look at their co-occurrence rate with proteins under investigation in this study. Medline is a source of citation and abstract information for biomedical literature. Natural language processing tools can be used to examine co-occurrence of relevant keywords to support hypothesis generation, such as in [3, 4]. Once repolarizing proteins are identified, they will be linked to signaling pathways using PPI tools. Tools that use the protein primary sequence and are capable of making interactome scale predictions are being investigated for this [5-7].

By performing co-occurrence analysis using VOSviewer, we have identified 461 entries where both of the IDs are genes that were found in our previous array experiments. New leads that are identified based on this approach will be built into the network described above. The next steps are to automate abstract collection for manual evaluation and to evaluate tools that identify any gene or protein name to expand the list. We are also considering methods for modeling how MEVs target the tumor and TAMs, such as in [8].

Figure 5: Summary of Cytoscape/NetworkX based network analysis.



Common Signaling Pathways from TLR, TNF, and IL-1b mediated pathway

Merged Pathway: TLR, TNF, IL-1b, and IL-6-mediated Pathways (source: Signor). These pathways are merged by Union

Table 1: M1 polarization pathways and their associated membrane receptors identified by the approach outlined in Fig. 1.

Rank	Receptor	Score
1	TNFR2 (TNFRSF1B)	82.592786
2	IL1R1	73.187031
3	CD40	56.207512
...	...	...
9	CD81	20.956046
10	CCL5/CCR3	7

Major Conclusions

- We have developed a computational model to identify key signaling networks responsible for proinflammatory macrophages
- Network analysis has identified TNFR2, CCR1, and CCR3 as top receptor candidates for promoting M1 polarization
- Automated literature searches are being investigated to predict proteins responsible for repolarization of M2 TAMs to M1 TAMs
- Co-occurrence analysis has identified 461 entries where both IDs are genes identified in our previous array experiments

b. Model validation and development of P-MEVs engineered to reprogram macrophages.

We have begun validating the candidate membrane receptors identified by network analysis in Objective 2a. Using western blot analysis, we have confirmed that CD40 is present on M1EVs, and the corresponding CD40 ligand (CD40L) is detected on M2 macrophages (Figure 6). We have also identified CXCL8 and CXCL10 as genes more highly expressed in human M1 macrophages compared to M0 and M2 macrophages (Figure 7). M2 macrophages treated with M1EVs exhibit elevated expression of CXCL8 and CXCL10, with CXCL8 expression reaching M1 levels.

We have initiated experiments with transfection of macrophages. These experiments were delayed due to troubleshooting methods to avoid cell toxicity from transfection reagents. We have identified reagents and methodology that limits this toxicity to acceptable levels. We are now proceeding with validation of top candidates identified from network analysis by overexpressing or knocking down the candidates in M1EVs and testing their ability to reprogram M2 macrophages.

Figure 6: The macrophage associated protein CD40 identified from network analysis is present on M1EVs.

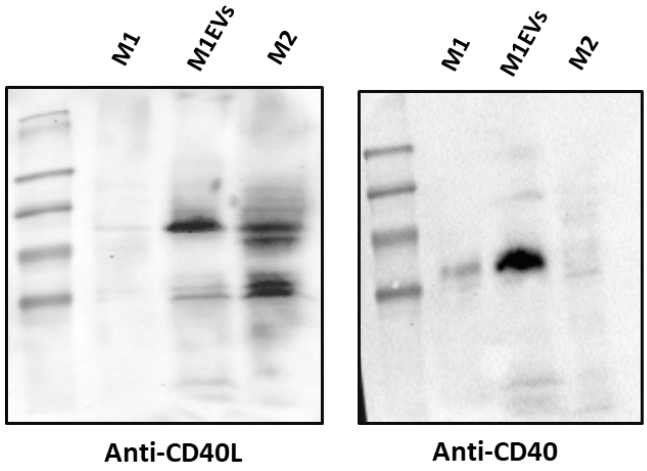
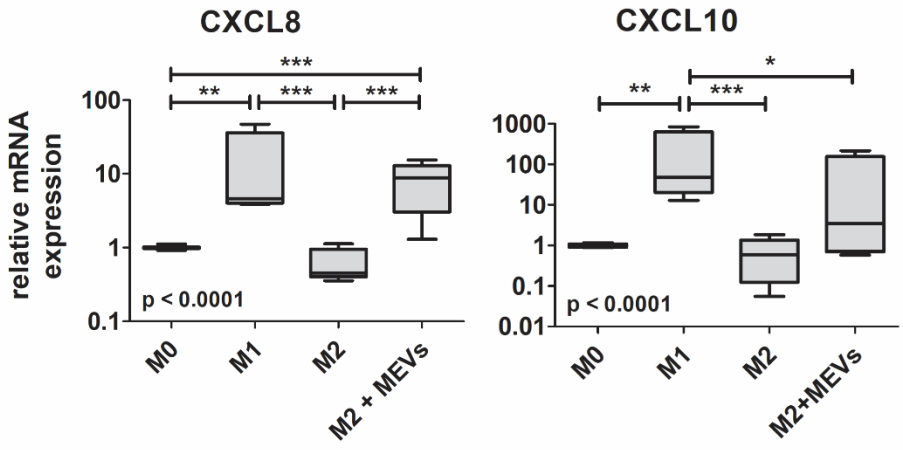


Figure 7: CXCL8 and CXCL10 are differentially expressed among human M1 and M2 macrophages



Major Conclusions

- We have validated that CD40, a top candidate identified from network analysis, is present on M1EVs
- CXCL8 and CXCL10 expression is significantly higher in human M1 versus M2 macrophages
- M1EVs induce CXCL8 and CXCL10 expression in M2 macrophages

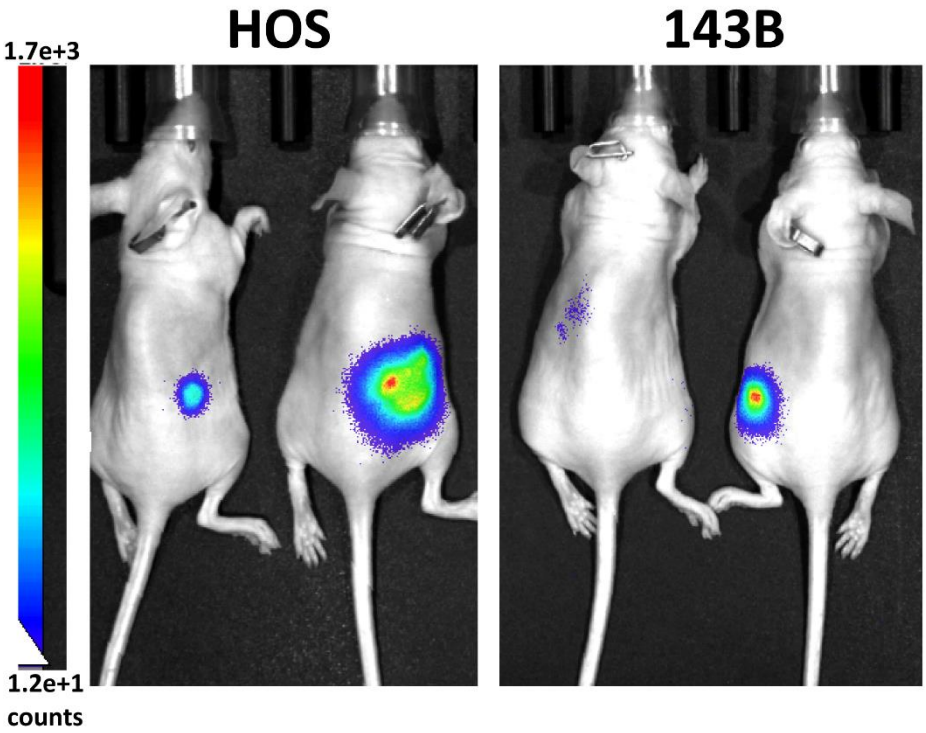
Objective 3: Determine the antimetastatic activity of P-MEVs in osteosarcoma xenografts.

a. To assess the antimetastatic activity of mouse bone marrow derived M1 vesicles.

This objective will rely on the development of the HOS and 143B osteosarcoma models. We have obtained HOS and 143B cells from ATCC. Initial plans were to create xenografts using intratibial injection of cultured cells in mice, however, this approach proved to be technically challenging in spite of training under the supervision of a DLAR veterinarian. Therefore, we have revised our experimental approach and will now establish subcutaneous xenografts in the flanks of nude mice.

In the previous quarter, we developed luciferase labeled HOS and 143B osteosarcoma cell lines, which will enhance monitoring tumor growth *in vivo* by allowing the use of bioluminescent imaging. These cells have been implanted subcutaneously in the flanks of nude mice and successful engraftment has been confirmed (Figure 8).

Figure 8: Bioluminescent imaging of luciferase-labeled HOS and 143B osteosarcoma xenografts



Major Conclusions

- We have obtained the 143B and HOS osteosarcoma cell lines and developed luciferase-labeled derivative lines
- Proof-of-concept subcutaneous xenografts have been established in nude mice using both luciferase-labeled 143B and HOS cells

b. Determine in vivo activity of cisplatin loaded vesicles.

These studies will begin in 2021.

c. Assess the in vivo targeting of MEVs derived from human monocytes in osteosarcoma xenografts.

These studies will begin in 2021.

References

[1] S. Dijkstra, E. E. Geisert Jr., C. D. Dijkstra, P. R. Bär, and E. A. Joosten, "CD81 and microglial activation in vitro: proliferation, phagocytosis and nitric oxide production," *J Neuroimmunol*, vol. 114, no. 1-2, pp. 151-9, 2001.

[2] W. Xuan, Q. Qu, B. Zheng, S. Xiong, and G.-H. Fan, "The chemotaxis of M1 and M2 macrophages is regulated by different chemokines," *J Leukoc Biol*, vol. 97, no. 1, pp. 61-69, 2015.

[3] S. Radhakrishnan, S. Erbis, J. A. Isaacs, and S. Kamarthi, "Novel keyword co-occurrence network-based methods to foster systematic reviews of scientific literature," *PloS one,* vol. 12, no. 3, pp. e0172778, 2017.
[4] N. J. Van Eck, and L. Waltman, "Text mining and visualization using VOSviewer," *arXiv preprint arXiv:1109.2058*, 2011.
[5] K. Dick, B. Samanfar, B. Barnes, E. R. Cober, B. Mimee, S. J. Molnar, K. K. Biggar, A. Golshani, F. Dehne, and J. R. Green, "Pipe4: Fast ppi predictor for comprehensive inter-and cross-species interactomes," *Scientific reports,* vol. 10, no. 1, pp. 1-15, 2020.
[6] Y. Li, and L. Ilie, "SPRINT: ultrafast protein-protein interaction prediction of the entire human interactome," *BMC bioinformatics,* vol. 18, no. 1, pp. 485, 2017.
[7] X. Liu, B. Liu, Z. Huang, T. Shi, Y. Chen, and J. Zhang, "SPPS: a sequence-based method for predicting probability of protein-protein interaction partners," *PloS one,* vol. 7, no. 1, pp. e30938, 2012.
[8] C. G. Cess and S. D. Finley, "Multi-scale modeling of macrophage-T cell interactions within the tumor microenvironment," PLoS Comput Biol, vol. 16, non. 12, pp. e1008519, 2020.

Deliverables (check appropriate time period when each deliverable is completed)	**Month 1-3**	**Month 4-6**	**Month 7-9**	**Month 10-12**	**Month 13-15**	**Month 16-18**	**Month 19-21**	**Month 22-24**	**√**
Notify DPH when IRB approval is received or if not required	N/A								N/A
#1 Amend the animal protocol and biosafety protocol to encompass experiments outlined in the proposal	√								7.13.2020 7.22.2020
#2 Recruit and train the graduate students and staff outlined in this proposal	√								9.30.2020
#3 Inhibit SLCO2B1 and SLC22A4 with chemical inhibitors and/or siRNA to assess the influence of SCLO2B1 inhibition on macrophage polarization	√								12.31.2020
#4 Screen for the specific proteins that are present on the surface of MEVs via microarray screening for 247 unique membrane proteins	√								12.31.2020
#5 Quantify receptors on the vesicle surface via quantitative flow cytometric analysis using antibody-phycoerythrin conjugates and Quantibrite phycoerythrin calibration beads									

#6 Inhibit identified membrane receptors through siRNA based gene silencing to determine their role in cell targeting and uptake									
#7 Conduct monthly meetings with Leadership team to discuss progress and problem-solve any potential barriers	√								7.9.20 8.5.20 9.2.20 10.7.20 11.4.20
#8 Submit a report outlining progress and any preliminary finding through December 31, 2020	√								12.2.20 1.15.2021
#9 Identify key molecular signaling networks responsible for MEV driven reprogramming of macrophages to a proinflammatory phenotype by computational modeling			√						4.8.2021
#10 Test model predictions on the regulation of protein expression and protein activity such as the phosphorylation of P38									
#11 Use a gene silencing approach to knock-down expression of specific components predicted from the modeling to differentially regulated M1-like versus M2-like phenotypes									
#12 Perform qPCR on target macrophages to identify changes in gene expression levels due to exposure to the P-MEVs				√					5.11.2021
#13 Generate KRIB and 143B osteosarcoma models				√					6.30.2021
#14 Treat four cohorts (saline control, cisplatin alone, MEVs alone and cisplatin + MEVs) of mice to assess efficacy and adverse effects									
#15 Conduct monthly meetings with Leadership team to discuss				√					1.6.2021, 2.11.2021,

progress and problem-solve any potential barriers									3.11.2021, 4.8.2021, 5.13.2021, 6.10.2021
#16 Submit a report outlining progress and any preliminary findings through June 30, 2021				√					7.15.2021
#17 Generate an adeno-associated virus (AAV) construct of the gene that codes for the target protein responsible for MEV re-programming									
#18 Assess the in vivo targeting of MEVs derived from human monocytes in osteosarcoma xenografts									
#19 Prepare first manuscript									
#20 Submit first manuscript									
#21 Conduct monthly meetings with Leadership team to discuss progress and problem-solve any potential barriers									
#22 Submit a report outlining progress and any preliminary findings through December 31, 2021									
#23 Assess the in vivo efficacy and adverse effects of MEVs derived from human monocytes in osteosarcoma xenografts									
#24 Prepare second manuscript									
#25 Submit second manuscript									
#26 Prepare grant submissions to continue research									
#27 Conduct monthly meetings with Leadership team to discuss									

progress and problem –solve any potential barriers. ***#28 Submit a report outlining progress and any preliminary findings through June 30, 2022***									

Quarterly Reports are due:

- October 15, 2020
- January 15, 2021
- April 15, 2021
- July 15, 2021
- October 15, 2021
- January 15, 2022
- April 15, 2022
- July 15, 2022

Reports should be returned to:
Janet.luttrell@ky.gov
Pediatric Cancer Program Manager
CHFS/DPH/Chronic Disease Prevention Branch
275 East Main Street, HS2WE
Frankfort, KY 40621