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TITLE: Targeting Metastasis by Inhibiting Breast Cancer Metabolism and Immune-Suppression

PRINCIPAL INVESTIGATOR: Jennifer Richer

CONTRACTING ORGANIZATION: University of Colorado, Aurora, CO

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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13. SUPPLEMENTARY NOTES

Triple negative breast cancer, tryptophan, immune suppressive, kynurenine, anchorage independent, aryl hydrocarbon receptor

14. ABSTRACT

Triple negative breast cancer (TNBC) has a particularly high rate of metastasis and the risk of recurrence after surgical removal of the primary tumor is within the first few years of initial diagnosis. A product of tryptophan breakdown called kynurenine is well-recognized as a mediator of immune suppression.. In TNBC the tryptophan catabolism pathway is induced by the enzyme tryptophan-2,3-dioxygenase (TDO2), not IDO1. TDO2 breaks down tryptophan to a product called kynurenine (Kyn), which protects tumor cells against cell death, but also when secreted from the tumor, is bad for the immune cells that should be killing tumor cells. Kyn was recently found to bind to receptors called aryl hydrocarbon receptors (AhR) in both tumor cells and immune cells and AhR promotes the ability of tumor cells survive in the bloodstream. We published that tryptophan depletion and AhR activation by Kyn decreases the survival and function of anti-tumor CD8+ T cells isolated from healthy people. We hypothesize that metabolic alterations in TNBC such as increased tryptophan catabolism are induced by anchorage independence and inflammation and result in the production of immune-suppressive metabolites, and that targeting these pathways will prevent or contain metastasis by boosting immune function.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:** We find that TNBC cells can rapidly change their metabolism to survive in the anchorage independent condition One of the things they change is the protein LAT1 that takes large amino acids such as tryptophan into the cancer cells. A product of tryptophan breakdown called kynurenine is well-recognized as a mediator of immune suppression. We found that programmed Cell Death Ligands PD-L1 and L2, which inhibit the function of anti-tumor CD8+ T cells, are also repressed by restoration of miR-200c. We hypothesize that metabolic alterations in TNBC such as increased tryptophan catabolism and heme catabolism by enzymes such as TDO2 and HO-1 are induced as a consequence of anchorage independence and inflammation, which result in production of immune-suppressive metabolites, and that targeting these pathways will prevent or contain metastasis by boosting immune function. Our **specific aims** are designed to 1) Identify the up- and downstream regulators/effectors of tryptophan catabolism to define what contributes to or supports metastatic potential and 2) determine how targeting tryptophan catabolism alters tumor infiltrating lymphocyte (TIL) composition or function in two immune competent models: syngeneic mouse mammary carcinoma models and *patient-derived xenografts (PDX)* in a "humanized" mouse model to test TDO2 inhibition alone or in combination with a PD1 checkpoint inhibitor. **Study Design**: We will determine whether inflammation- and anchorage independent survival induce TDO2 and other enzymes that produce immune-suppressive catabolites or mimic immune cells to evade immune attack. We will identify up and downstream effectors of TDO2 in BC cells and patient derived xenografts and we will trace tryptophan catabolism in TNBC under conditions that affect TDO2 levels and activity. We will take our prior study further towards the clinic by targeting TDO2 in TNBC patient-derived xenografts (PDX) in a "humanized" mouse model and test this strategy alone or in combination with checkpoint inhibition.

2. **KEYWORDS:** triple negative breast cancer tryptophan, heme oxygenase, tumor infiltrating lymphocyte

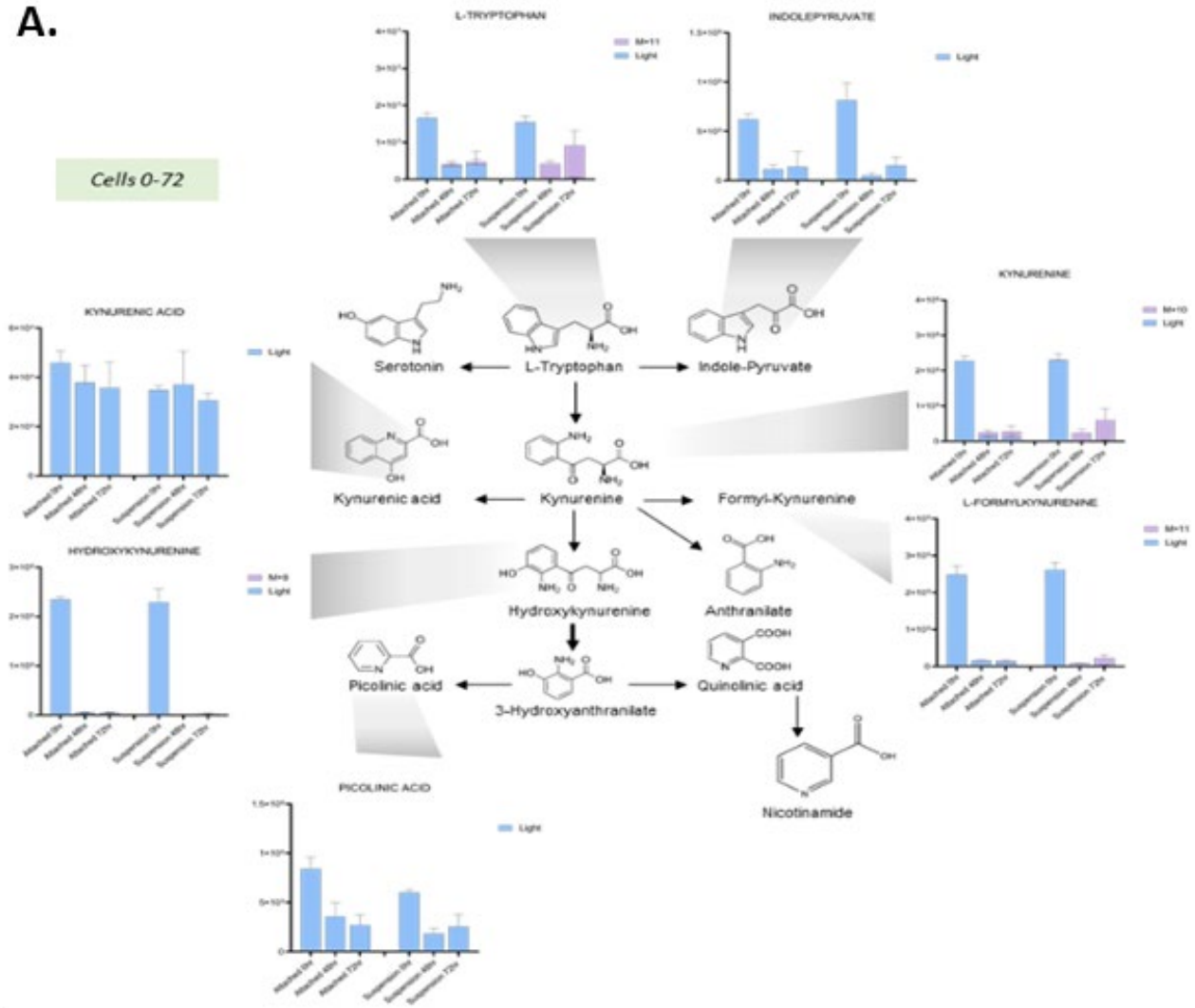
3. **ACCOMPLISHMENTS:** The major goals of the project listed in the SOW are below along with what was accomplished this past year.

Aim 1. Identify the up- and downstream regulators/effectors of TDO2 to define what contributes to or supports metastatic potential. (Months 1-12)

Task 1. Tryptophan tracing with metabolomics core in TNBC under conditions that affect TDO2 levels and activity. (Months 1-8) 100% completed. Prior work in our lab and others established the immunomodulatory and pro-tumorigenic functions of kynurenine. However, it remains to be elucidated how TNBCs utilize this pathway under different conditions and how much tryptophan is used towards the production of other tryptophan metabolites. We had previously reported that the enzyme TDO2 that catabolizes tryptophan in breast cancer cells increases in suspension culture (anchorage independent condition modeling metastasis) and also with inflammatory cytokines that are known to activate NFκB, mimicking inflammation. Tracing of labeled tryptophan was performed over time (to observe the flux of the tryptophan) as proposed in this aim in order to determine if TNBC preferentially produce specific pathway metabolites. This task is now complete and we observe that ***^{13}C*** labelled tryptophan is taken up in the cells at 48 and 72 hours more in suspension culture and is converted into kynurenine in the suspended (anchorage independent) condition (**Figure 1**). The heavy tryptophan was also primarily metabolized by the tumor cells into to kynurenine and L-formylkynurenine and secreted into the media as well (not shown). We also conducted the tracing experiment after treatment with inflammatory cytokines (IL1beta and TNFalpha) that activate NFkB (**Figure 2**). For the tracing cells were incubated in DMEM L-tryptophan depleted media (MBS653056, MyBioSource) supplemented with 0.016 mg/l ^{13}C11 labeled L-Tryptophan (99% CLM-4290, Cambridge Isotope Laboratories, Inc) for the times and treatments indicated in the figure legends. Both cells and media were harvested and metabolomics analyses performed via UHPLC-MS. These results as well as how a new inhibitor that targets both IDO and TDO2 affects kynurenine production and other metabolites (**Figure 3**) are reported below. We are in the process of writing a manuscript on these results and also determining how the inhibitor affects growth on soft agar and metastatic potential.

We also met our **milestone** scheduled to be accomplished by month 8, which was to obtain IACUC and ACURO approval for the animal protocol. This milestone was **achieved and the IACUC and ACURO approvals are included in the appendix.**

Figure 1. Tracing of ^{13}C labeled tryptophan demonstrates that tryptophan uptake and catabolism increases when TNBC cells are cultured under anchorage independent conditions. *MDA-MB-453 were cultured in attached and suspended conditions and harvested at 0, 48 and 72 hours after addition of ^{13}C labeled tryptophan to the cells cultured in tryptophan depleted media .* ***A.*** *Heavy ^{13}C labeled carbon from tryptophan in the breast cancer cells is shown in purple and unlabeled residual tryptophan is shown in blue.* ***B.*** *The same data is depicted with just the heave labelled carbon shown to more easily visualize the increase in uptake of tryptophan over time which was significantly increased over time in the suspension culture as compared to the traditional attached cell culture conditions. Two-way ANOVA was performed *: p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*



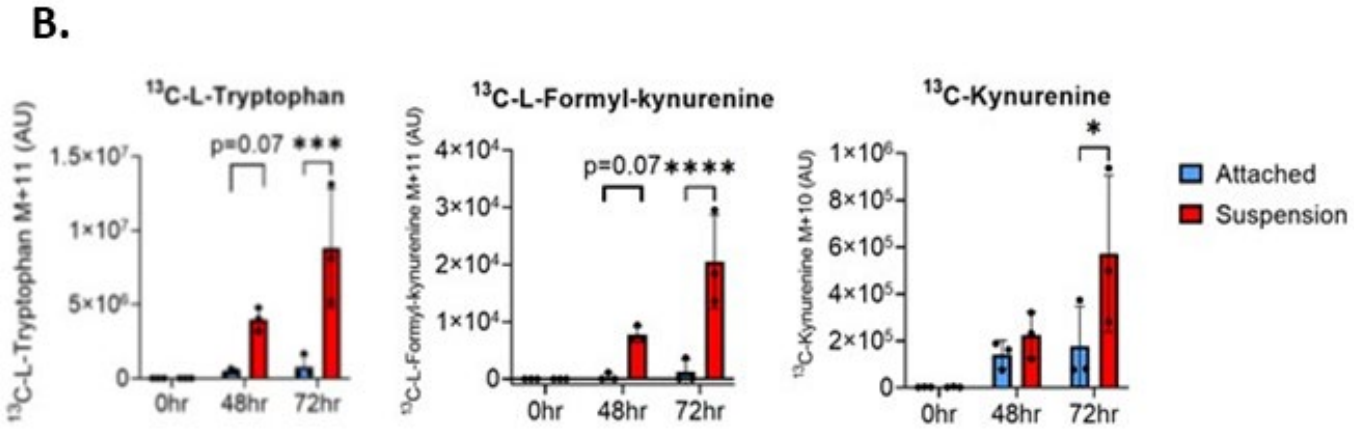


Figure 2. Tracing of ^{13}C labeled tryptophan demonstrates that tryptophan uptake and catabolism increases when TNBC cells are treated with NFkB activating cytokines over time in attached versus anchorage independent conditions. *NFkB activating cytokines increase the influx of the early stage of tryptophan catabolism pathway and accumulate specific metabolites. MDA-MB-453 were cultured in regular attached and suspension (poly-hema coated plates) then treated with TNFα and IL1β. The C13 labeled tryptophan was added. By Two-way ANOVA defined *: p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

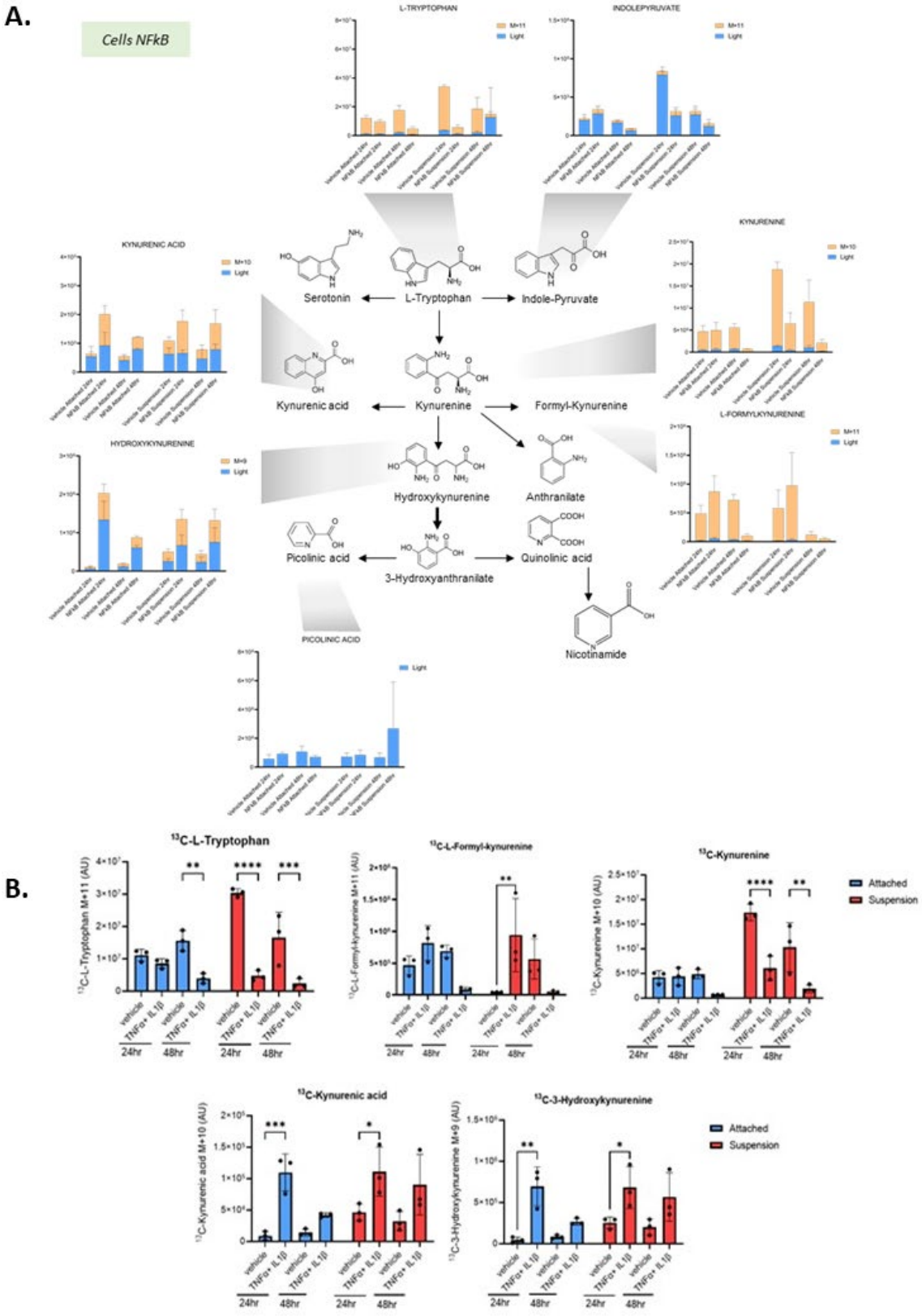
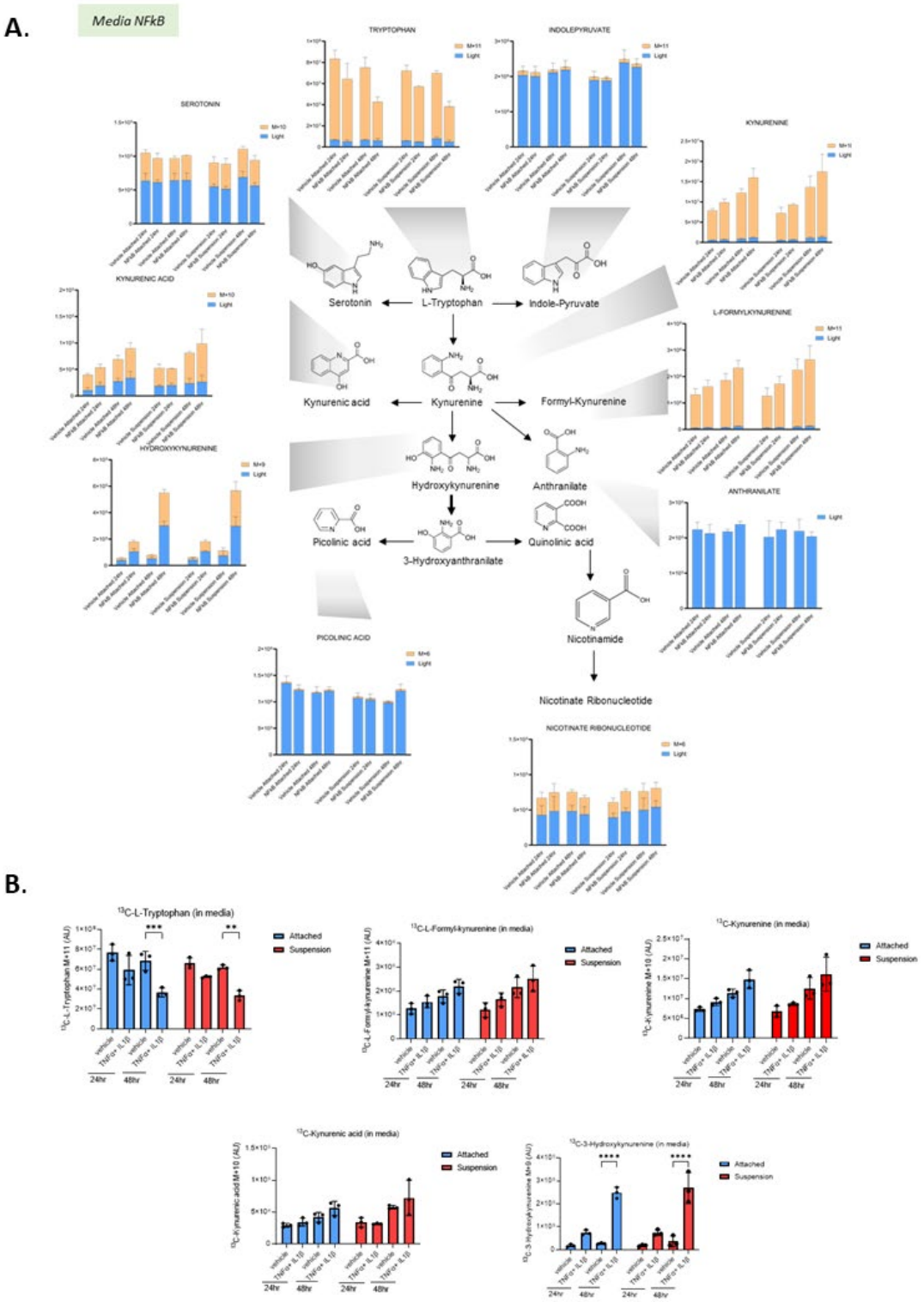


Figure 3. Media labeled tryptophan is depleted when NFkB is activated and over time more of the downstream metabolites kynurenine and L formylkynurenine are detected in the media A. *Both heavy labeled carbon (orange) from tryptophan and the light form in blue are depicted as detected in each metabolite* ***B.*** *Only the heavy carbon is plotted to visualize how much tryptophan was depleted and predominant downstream metabolites produced and secreted into the media over time in both the attached and suspended conditions*

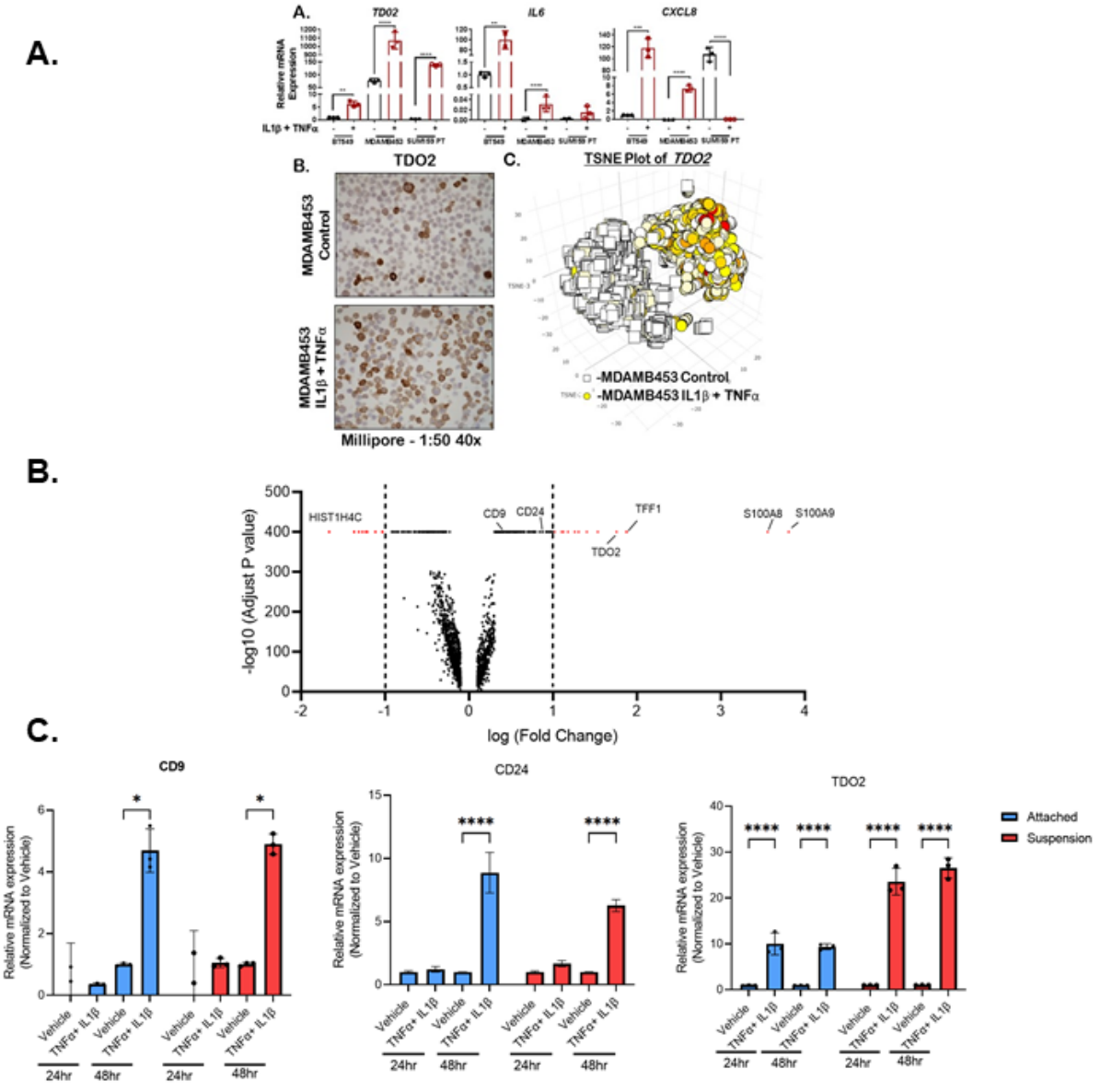


Task 2. Test changes in tryptophan catabolism and other metabolic changes following separation of TDO2+ vs negative cells by flow cytometry in MDA-MB-453 cells following induction of TDO2 with anchorage independent growth selection or IL1beta/TNFalpha using Seahorse Bioanalysis. Do the same in additional

TNBC cells (SUM159 and BT5249) and following genetic or pharmacologic inhibition of TDO2 or AhR levels or activity. **(Months 1-8).**

We are working to confirm that two genes *CD9* and *CD24*, that encode cell surface proteins tracked with the amount of TDO2 in the RNA-seq data that we showed in the grant because we want to use them for flow cytometry to sort out cells positive for TDO2 protein since not all of the cells even within a cell line express TDO2 (Figure 4 A and B). We confirmed by qRT-PCR that these two genes do correlate with TDO2 expression and like TDO2, are regulated by inflammatory cytokines (**Figure 4C**) so now we are selecting antibodies that we can use for flow cytometry to pull out cells enriched for high TDO2 to accomplish Task 2. We will also see if the secreted protein Trefoil Factor 1 (TFF1) would be useful for this purpose as well since it also tracked with TDO2.

Figure 4. TDO2 mRNA increases in TNBC cell lines following NFkB stimulation and the cell surface markers CD9 and CD24 are co-expressed in TDO2 high cells. A. *BT549, SUM159 (400,000 cells/well) and MDAMB453 (600,000 cells/well) were plated in 6 well dishes and treated 24 hrs later ± 10 ng/mL IL-1beta and 10 ng/mL TNFα. mRNA was harvested after 24 hours treatment. TDO2 expression and NFkB pathway activation (CXCL6, IL6 and CXCL8, IL8) were analyzed by qRT-PCR.* ***B.*** *MDA-MB-453 cells treated with 10 ng/mL IL1beta + 10 ng/mL TNFalpha or vehicle control were harvested after 24 hours and TDO2 IHC performed.* ***C.*** *Over 3,000 cells were captured/sample and sequenced at a depth of ~70,000 reads/cell. TSNE plot generated to observe TDO2 expression fafter stimulation (square = control treated cells; circle = IL1β + TNFα treated cells).* ***B.*** *A volcano plot shows that the genes CD9 and CD24 and TFF1 increase in a statistically significant manner along with TDO2 in the MDA,-MB-453 cells.*

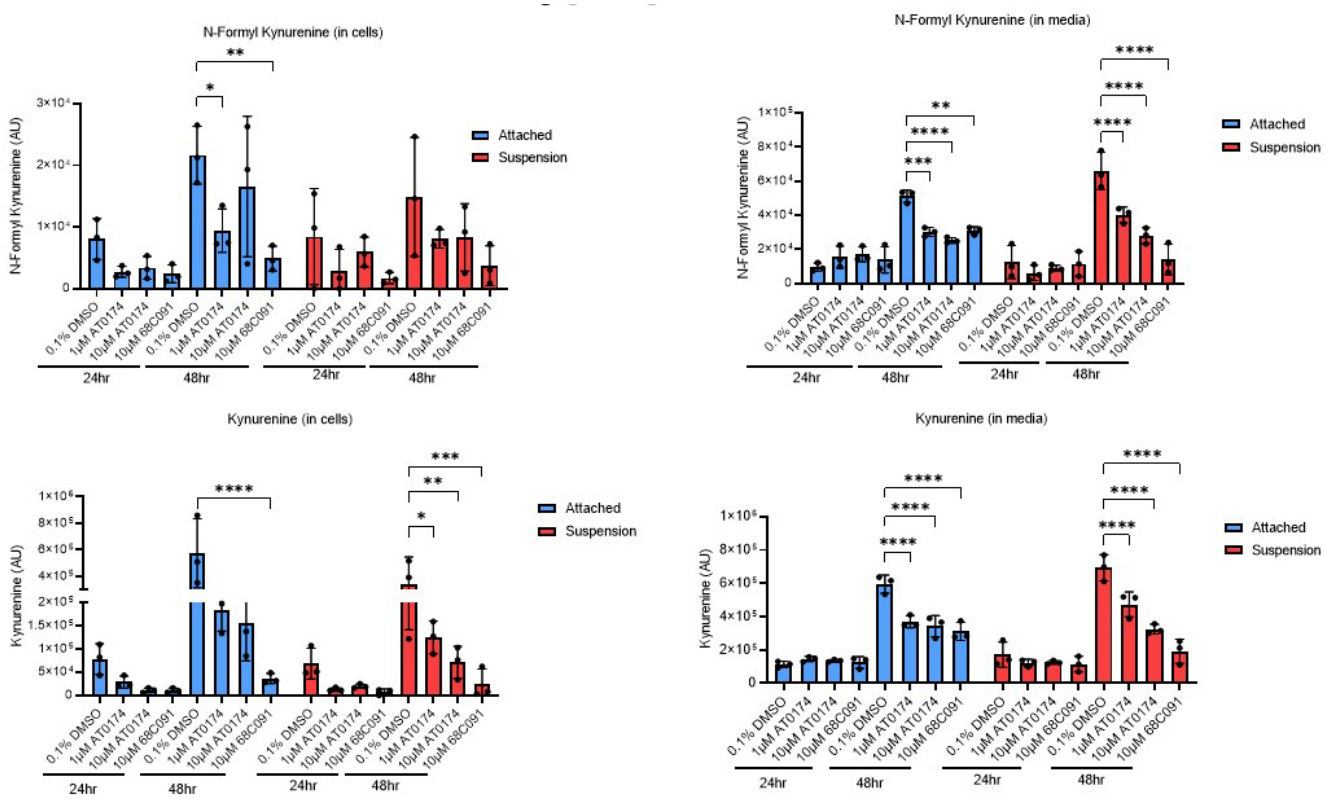


Task 3. Determine if cells isolated by flow cytometry for markers of TDO2 positivity are more able to form mammospheres and are more tumorigenic as determined by limiting dilution tumor formation assays in vivo. Use MDA-MB-453, SUM159 and BT5249). **(Months 6-12)** (**not yet completed** until we find the best way separate out the TDO2 high cells by flow cytometry in Task 2).

Task 4. Test drugs targeting the tryptophan catabolism pathway by different methods (TDO2 inhibitor (Antido), AhR inhibitors or and kynase enzyme inhibitor (Kyn-400) (Kyn Therapeutics) in TNBC cell lines and cells isolated from two PDX models (PK49 and HCI-009) in vitro to determine which method of inhibiting the tryptophan catabolism pathway results in best inhibition of tryp uptake, Kyn production and AhR activation. **(Months 6-12) (50% completed)**

Two different TDO2/IDO inhibitors AT0174 and 68c091 (tenatoprazole) that reduce production of immune suppressive tryptophan catabolites made by cells (left) and also reduced the levels of secreted catabolites (right) detected in the media (**Figure 5**).

Figure 5. Two different TDO2 inhibitors decreased breast cancer production of tryptophan catabolites in a dose dependent manner both in the attached and anchorage independent (suspended) condition as determined by mass spectroscopy. *Both TDO2 inhibitors, AT0174 and 68c091 (tenatoprazole), were tested in MDA-MB-453 breast cancer cells in both the attached (blue) and suspended (red) conditions and the amounts of N-formyl kynurenine (top) and kynurine (bottom) were quantified by mass spec both in the cells (left side)*

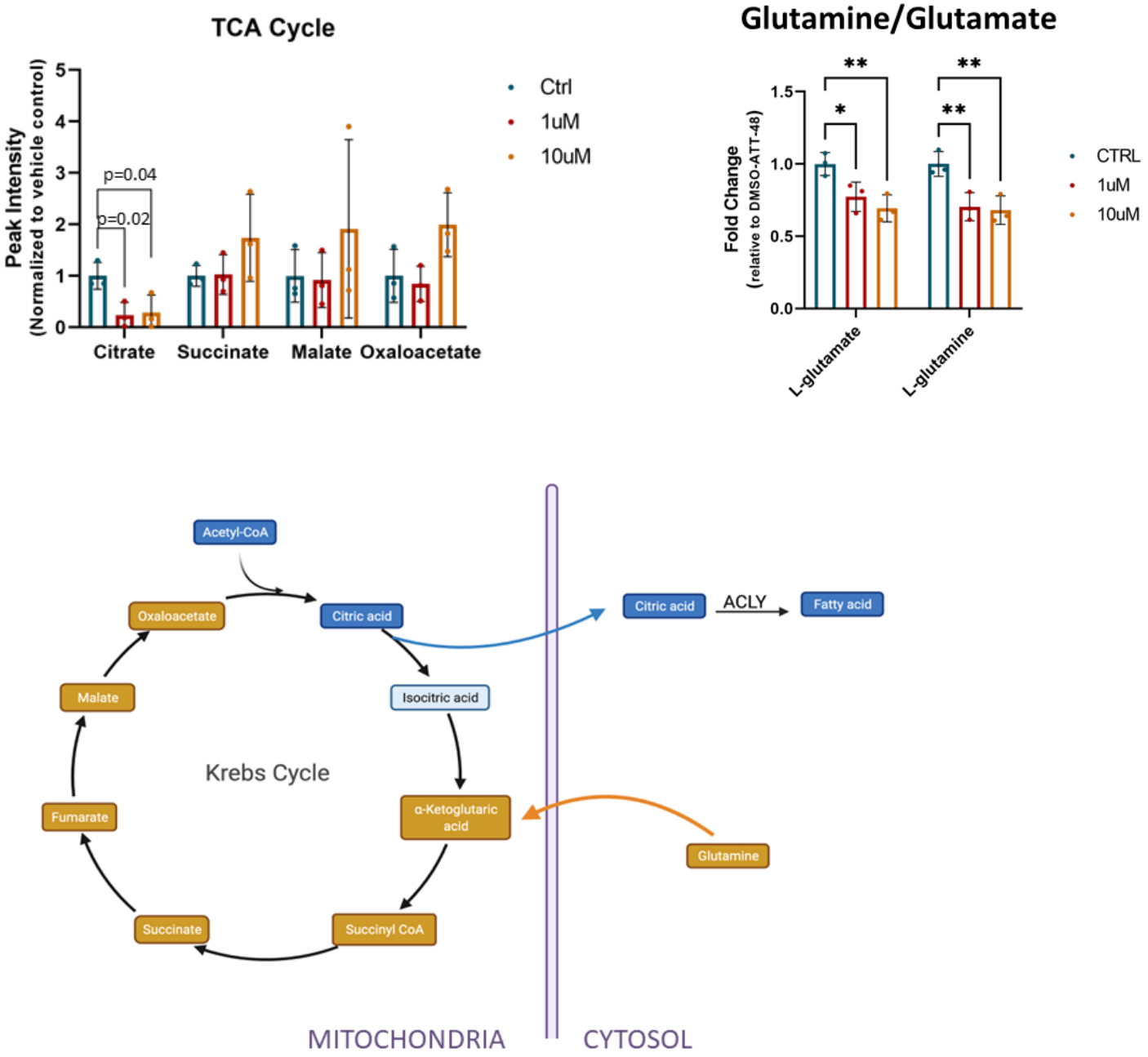


and in the media (right side).

We also find that the Antido TDO2-IDO inhibitor drug AT0174 decreases citrate in the TCA cycle in a dose dependent manner. This effect was unanticipated, but since we performed the 200 metabolite panel by However, the later steps of the cycle generating succinate, malate and oxaloacetate were not significantly altered (**Figure 6**). One explanation for this is that citric acid is being used up elsewhere and not progressing through TCA. This could possibly through the citrate-malate shuttle that uses it for de novo lipogenesis. Glutamate gets taken up by the cell and converted into glutamine. We observe a decrease in glutamate and glutamine with the TD02 inhibitor

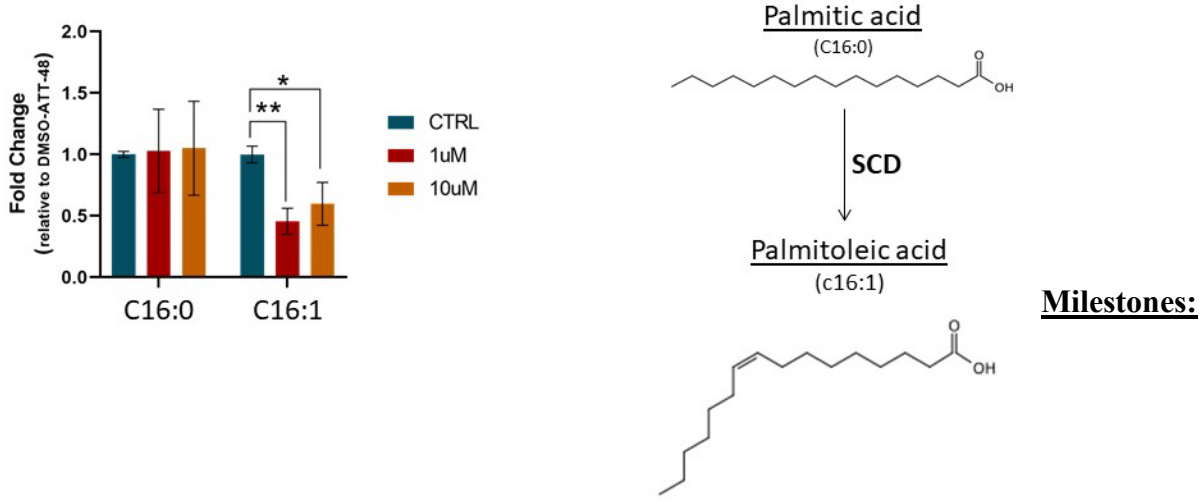
at two different doses. Cells treated with drug may be converting it into a-ketoglutarate for the TCA. The same results were obtained for the TCA cycle and glutamine and glutamate with both the AT017 and older 68c091 TDO2 inhibitor that is less optimal for in vivo experiments. A model (Figure 6 bottom) summarizes the conversion of glutamine through the TCA into citrate that may be used to make fatty acids.

Figure 6. Effects of the TDO2-IDO inhibitor AT0174 on other aspects of metabolism as detected by UHPLC-MS via a 200-metabolite assay. *Inhibition of tryptophan catabolism affected the amount of citrate inside MDA-MB-453 cells after 48 hours at two different concentrations (1 and 10 microMolar) as compared to vehicle control. Howeverr, the other metabolites in the TCA cycle were not affected, suggesting that perhaps the citrate was being shunted into making fatty acids as a fuel source. The lower levels of glutamine following TDO2 inhibition further suggests that the TCA cycle is being replenished by the conversion of glutamine to a-ketoglutarate.*



The TDO2 inhibitor AT0174 also reduces the amount of palmitoleic acid detected inside the cells (**Figure 7**).

Figure 7. AT0174 suppresses production of palmitoleic acid produced by* Stearoyl-Coenzyme A Desaturase 1 (*SCD). *Both palmitoleic acid and SCD expression correlate with tumor aggression and metastasis in patients. Surprisingly, we find that TDO2 inhibition led to a decrease in palmitoleic acid through mechanisms that we are continuing to explore. Decreased palmitoleic acid in these cells could have further anti-tumor effects, including those related to immune signaling.*



Milestones:

Milestone: Analyze data from tasks 1 and 2 and write manuscript (by Month 12)
Publish manuscript on altered metabolism and tumorigenic properties associated with TDO2 (Months 8-12) 50% complete. We are currently writing up the manuscript. We were set back somewhat by still being in shifts in the lab during this reporting period, but feel that we have made good progress.

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

Kathleen O'Neill, a 6th year student in the Cancer Biology Graduate Program has an NCI F99 grant on a different metabolism topic in the lab, but she has been involved in the tryptophan catabolism project since early on, but is not supported by this grant. Graduate student Li-Wei Kuo in the Cancer Biology Graduate Program rotated in the lab in the fall of 2020 and joined the lab in June of 2021. In July he was put on this DOD Expansion Award. Li-Wei performed the tryptophan tracing experiments with our Metabolomics shared resource core in our Cancer Center and is learning more about metabolic flux and metabolism in general. He also performed the RT-PCR for CD9 and CD22. He is presenting a poster at our Cancer Biology Graduate Program Retreat in November and we are working on the manuscript that he and Katie O'Neill will be co-first authors on. A postdoctoral fellow, Lyndsey Crump, was also hired in August 2021 on this grant to help with the effects on the immune system and in vivo experiments in the subsequent proposed Aims during year 2 and 3. She and another postdoctoral fellow Michelle Williams are working on immune suppressive factors like Heme Oxygenase (HO-1) that generate immune suppressive metabolites (similar to the tryptophan catabolism generating kynurenine in this project.

- **How were the results disseminated to communities of interest?** Nothing to report due to covid impeding community engagement quite a bit.
- **What do you plan to do during the next reporting period to accomplish the goals?**

Net we will determine if we can pull out the breast cancer cells that are expressing TDO2 by flow cytometry using CD9 or CD24 in order to start to perform the Seahorse metabolic assays in Tasks 3 and 4.

IMPACT:

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

Nothing to Report during this funding period.

- **What was the impact on other disciplines?**

  We are finding that a new inhibitor of the enzyme TDO2 block production of the immune suppressive catabolite kynurenine. This affects the field of tumor immunology.

- **What was the impact on technology transfer?**

  - Nothing to Report during this funding period.

- We are finding that a new inhibitor of the enzyme TDO2 block production of the immune suppressive catabolite kynurenine so this data along with future in vivo data may lead to clinical trials with the TDO2 inhibitor ***AT0174.***

- **What was the impact on society beyond science and technology?**

  - Nothing to Report

CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

- We are not changing the approach but we examined whether either kynurenine (that we published previously to affect cytotoxic T cell function) or another secreted metabolite bilirubin affect macrophages in vitro. So far the billirubin did affect macrophage fuction, but kynurenine did not. Later we will determine how these secreted catabolites affect both T cell and macrophage infiltration in vitro.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

- A bit less progress due to having to do shifts still with people in the lab only at 50% capacity in 2020 and spring of 2021 up until June 2021.

- **Changes that had a significant impact on expenditures**

- Li-Wei Kuo and Dr. Crump did not start until July and August respectively due. Prior to that Li-Wei was a rotation student so was paid for by the department.

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

  The ACURO approval is included in the appendix. The IACUC approval was on May 20, 2021 and the ACURO approval came through on July12, 2021

- **Significant changes in use or care of human subjects none**

- **Significant changes in use or care of vertebrate animals. none**
- **Significant changes in use of biohazards and/or select agents. none**

5. **PRODUCTS:**
   - **Publications, conference papers, and presentations**

     - **Journal publications.** Nothing to Report
     - **Books or other non-periodical, one-time publications.** Nothing to Report
     - **Other publications, conference papers, and presentations.**

Sept 2020 **Northwestern University Feinberg School of Medicine Department of Pharmacology**- "Targeting tryptophan catabolism in breast cancer."
April 2021* **American Society of Biochemistry and Molecular Biology** – Signaling in Breast and Ovarian Cancer Interest Group "Breast and ovarian cancers co-opt mechanisms of immune suppression used during pregnancy
May 2021* **Buenos Aires Breast Cancer Symposium**: From hormone receptors to the immune system. "Breast cancer hijacks a trophoblast-like program of immune suppression."

   - **Website(s) or other Internet site(s)** None to date
   - **Technologies or techniques**. None to date
   - **Inventions, patent applications, and/or licenses** None to date
   - **Other Products**

     - *data or databases; data from single cell seq of TNBC MBA MB 453 cells high and low for TDO2 before and after stimulation of NfKB deposited in our CU Cancer Center Shiny apps program.*

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

	Julie Lang
Project Role:	*collaborator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0

Contribution to Project:	*Advising on which type of humanize mice to use in future*
Funding Support:	
	Kathleen Torkko
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Advising on statistics
	Dr. Jennifer Richer
Project Role:	Principal investigator supervisor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	**2.0**
Contribution to Project:	Oversees experimental design
	Jill Slansky
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Advising on tumor immunology experiments and flow cytometry

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to report
    - **What other organizations were involved as partners?**
- **SPECIAL REPORTING REQUIREMENTS**
    - **COLLABORATIVE AWARDS:**
    - **QUAD CHARTS:**
- **APPENDICES:**

DEPARTMENT OF THE ARMY

HEADQUARTERS, U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000
July 15, 2021

Director, Office of Research Protections
Animal Care and Use Review Office (ACURO)

Subject: Approval of Proposal Number BC191031, Award Number W81XWH-20-1-0617 entitled, "Targeting Metastasis by Inhibiting Breast Cancer Metabolism and Immune-Suppression"

Dr. Jennifer Richer, PhD
University of Colorado Denver
Denver, CO, US

Dear Dr. Jennifer Richer, PhD:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Conducted and Supported Research and Training"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"

In accordance with the above references, ACURO protocol BC191031.e001 entitled, "Targeting Metastasis by Inhibiting Breast Cancer Metabolism and Immune-Suppression.," IACUC protocol number 01056, Protocol Principal Investigator Dr. Jennifer Richer, PhD, is approved by ACURO as of 07/12/2021 for the use of mice and will remain so until modification, expiration or cancellation. This protocol was approved by the University of Colorado Denver IACUC on 05/20/2021; IACUC approval expires 05/20/2024.

Required Actions:

A. Submit to ACURO for review and approval prior to implementing:

• IACUC-approved de novo reviews of the protocol

• IACUC-approved significant changes to this protocol (see guidance document)

B. Notify ACURO within 5 business days of any of the following:

• Any noncompliance, suspensions or adverse events (see guidance document)

• Receipt of notification that the institution is under investigation by USDA

• AAALAC, International accreditation status change

For further assistance, please contact ACURO at (301) 619-6694, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

Krinon Moccia, DVM, MPH, DACLAM
LTC, VC, USA
Director, Animal Care and Use
Review Office

Copies Furnished:
Garrett Steed
Dr Jori Leszczynski
Dr. Mark Douse
Dr. Jennifer Richer, PhD
Dr. Jessica Brusgard
Dr. Ashley Schneekloth
Michael L. Moore