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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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Jennifer K. Richer, Ph.D.

E-Mail: Jennifer.richer@cuanschutz.edu

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University of Colorado
Anschutz Medical Campus
12800 E. 19th Ave
Aurora, CO 90010

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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.

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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

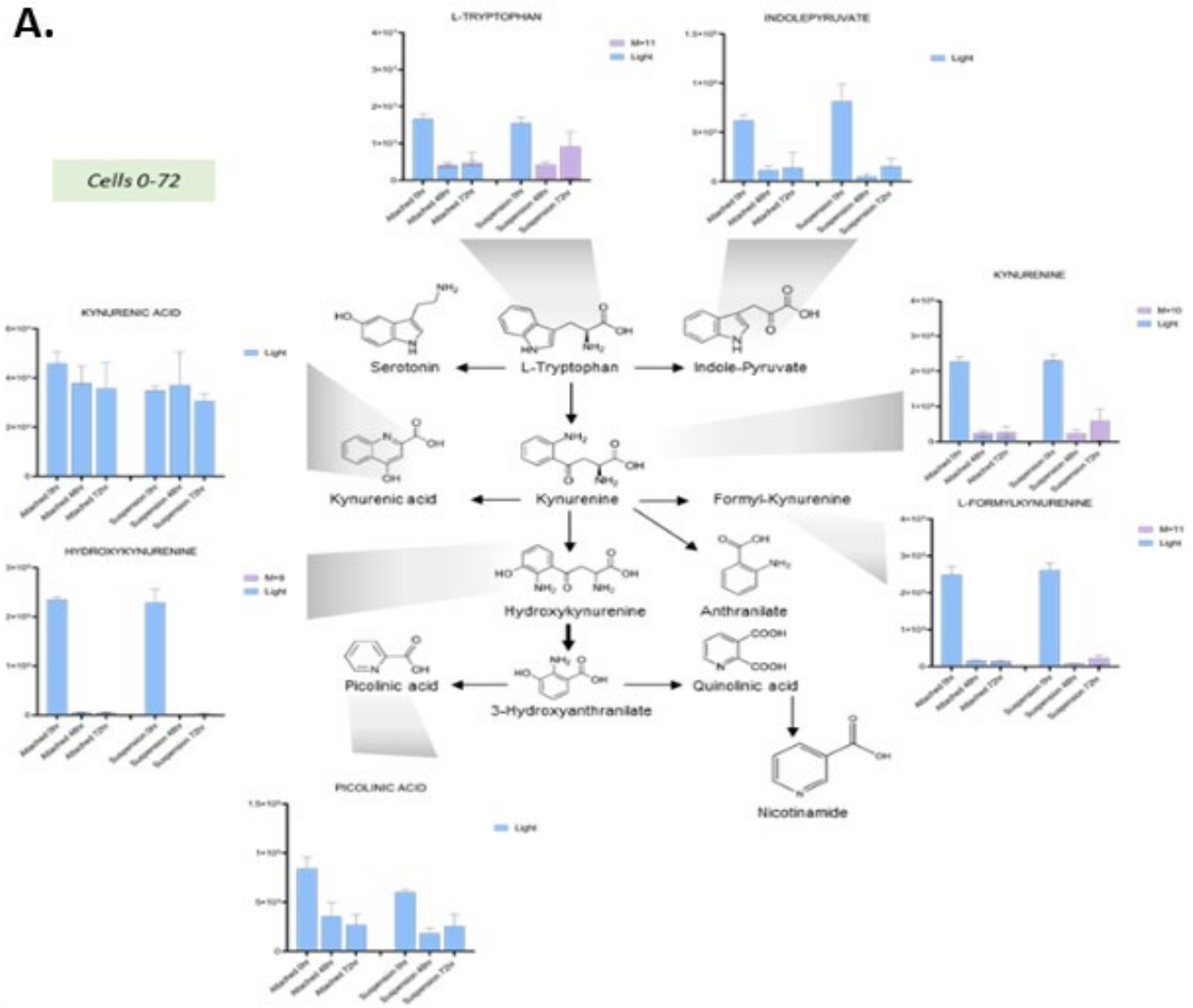
3. **ACCOMPLISHMENTS:** The major goals of the project listed in the SOW are below along with what was accomplished this past year.

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% complete. 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 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.*



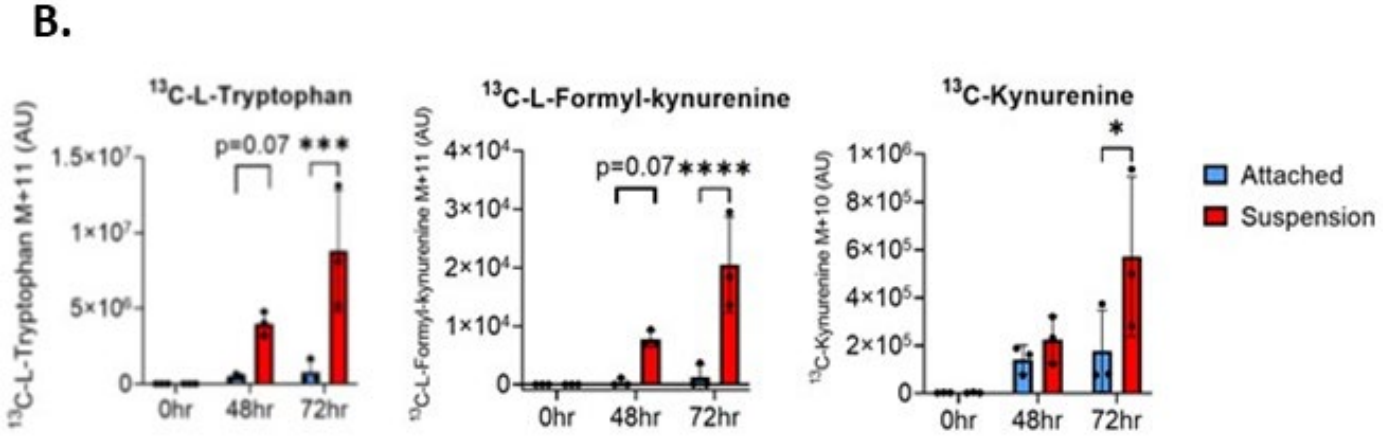


Figure 2. Tracing of ^{13}C labeled tryptophan demonstrates that tryptophan uptake and catabolism increases when TNBC cells are treated with NFkB activating cytokines over time in attached versus anchorage independent conditions. *NFκB activating cytokines increase the influx of the early stage of tryptophan 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.*

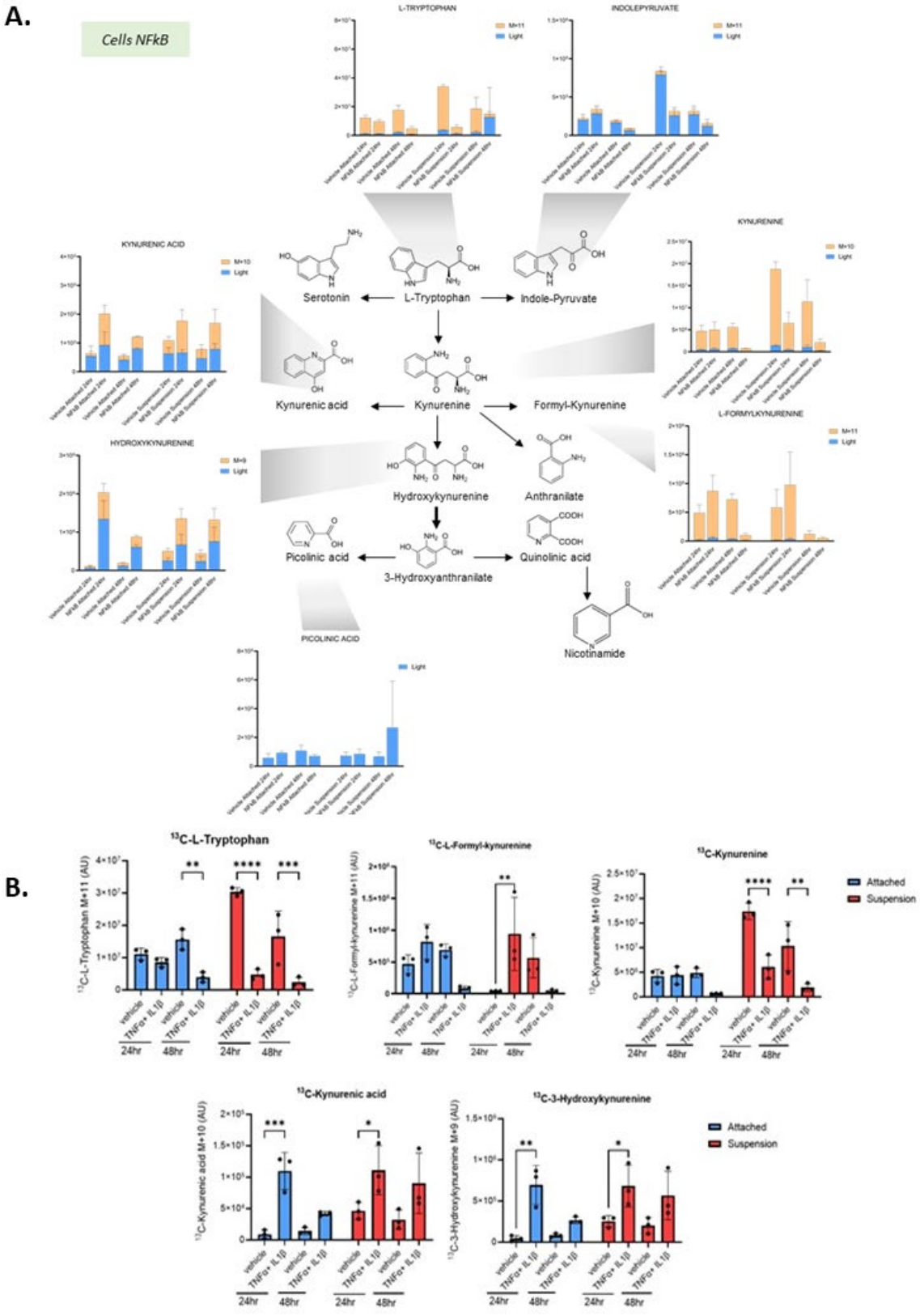
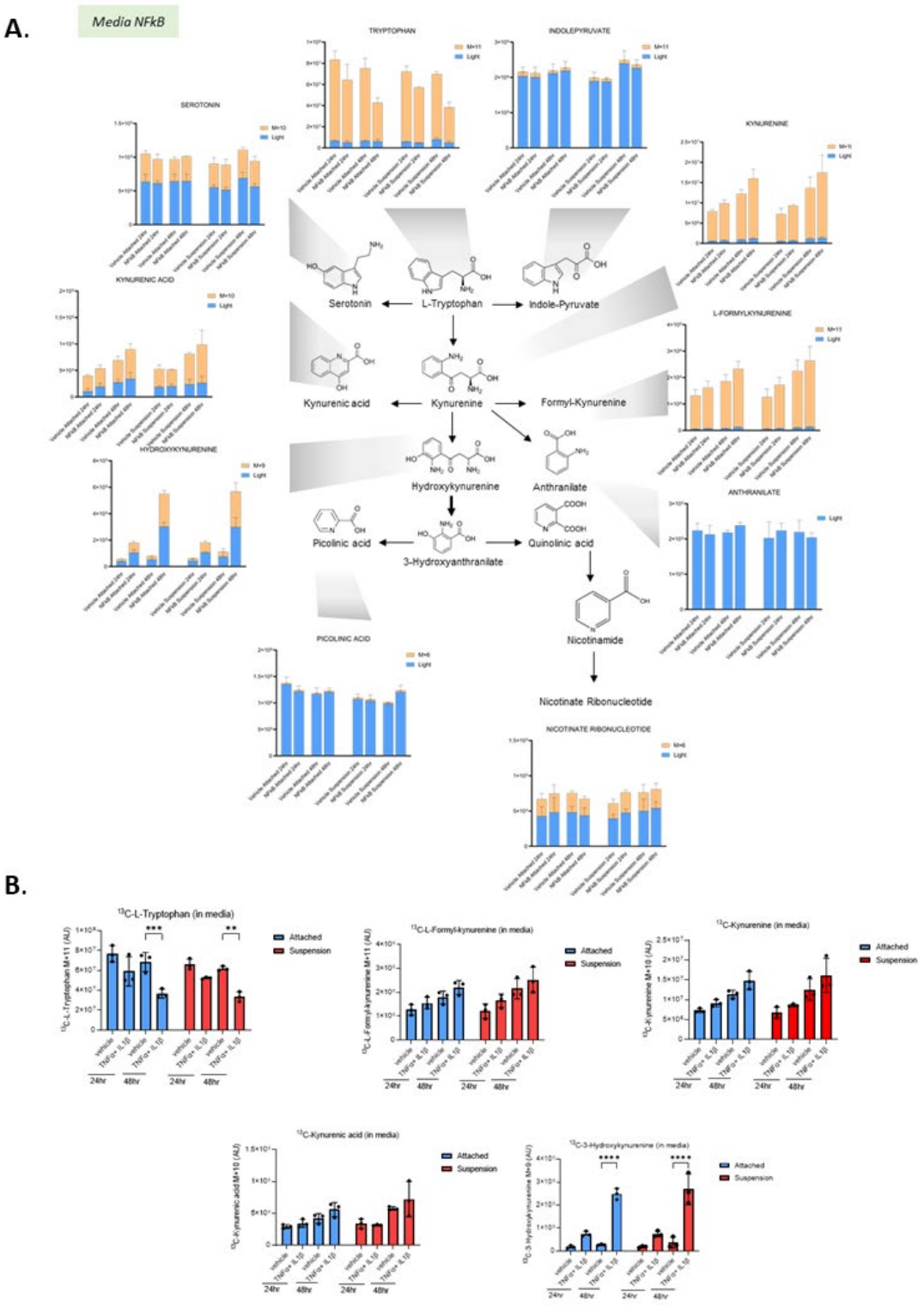


Figure 3. Media labeled tryptophan is depleted when NFkB is activated and over time more of the downstream metabolites kynurenine and L formylkynurenine are detected in the media A. *Both heavy labeled carbon (orange) from tryptophan and the light form in blue are depicted as detected in each metabolite* ***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*

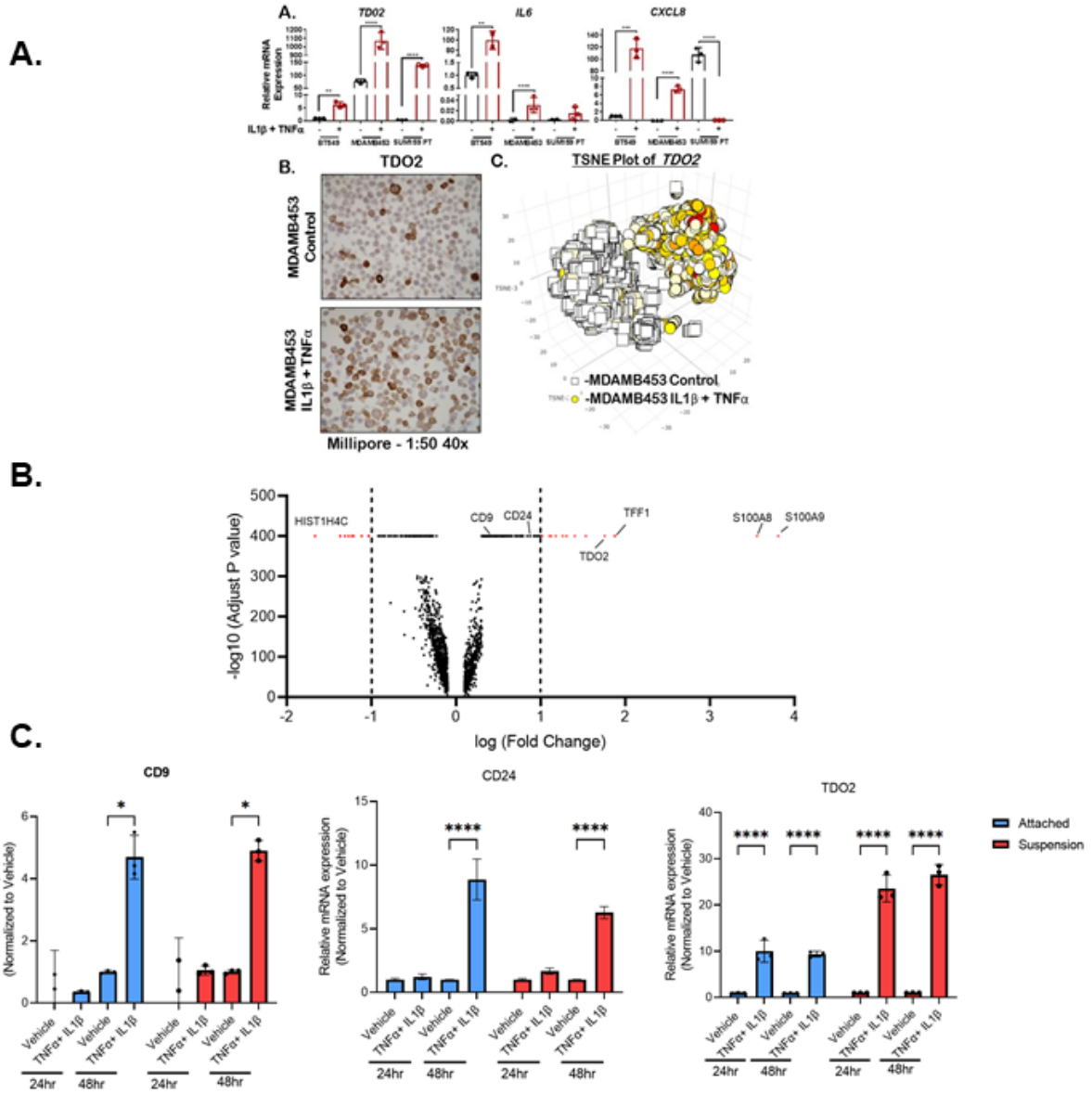


Task 2. Test changes in tryptophan catabolism and other metabolic changes following separation of TDO2+ vs negative cells by flow cytometry in MDA-MB-453 cells following induction of TDO2 with anchorage

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). (75% completed)**

We confirmed that two genes *CD9* and *CD24* came up in our original data (Figure 4 A and B) that encode cell surface proteins tracked with the amount of TDO2 in the RNA-seq data. These correlated with TDO2 levels and that we could use them to select out TDO2 positive cells. 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 may 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.*



However, we decided not to use them for flow cytometry to sort out cells positive for TDO2 protein since they were more broadly expressed than TDO2. We found that selecting by flow cytometry for cells that survive 48 hours in anchorage independent culture on low adherence plates for 48 hours are enriched for expression of *TDO2*.

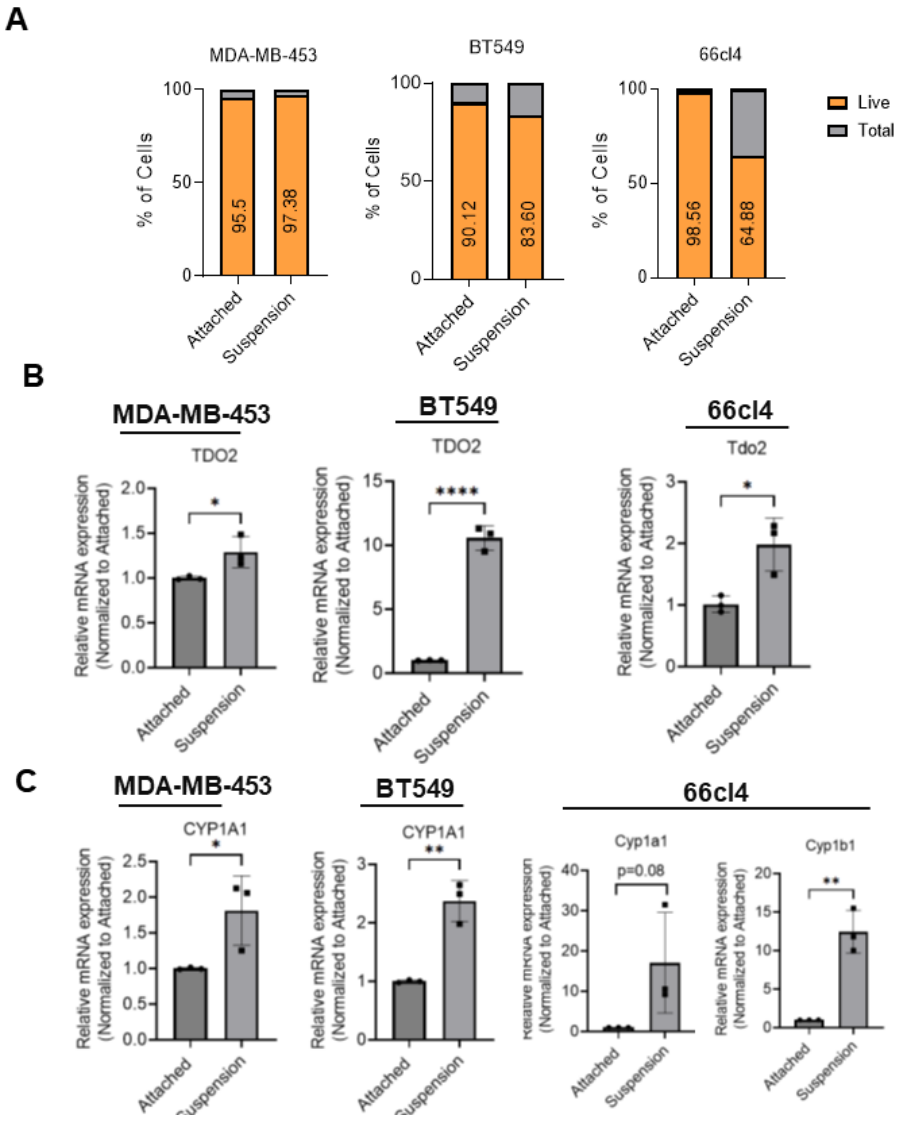
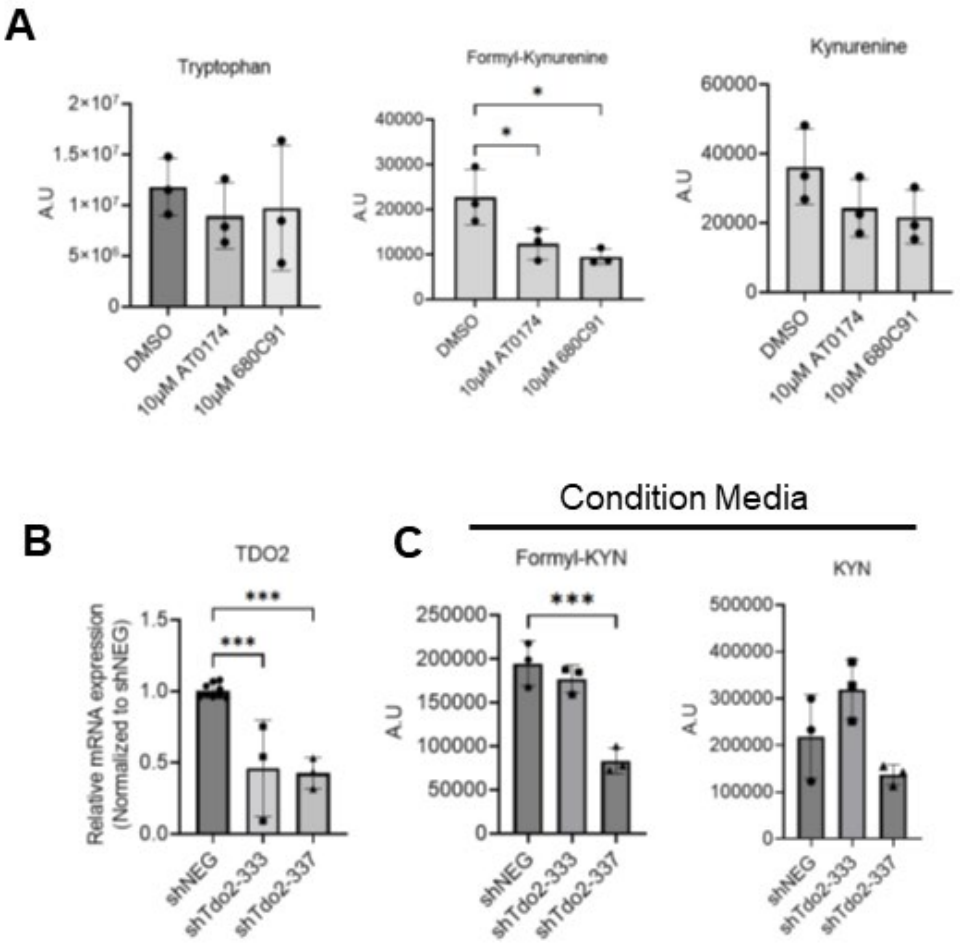


Figure 5. TNBC cultured for 48 hours in anchorage independent condition have enriched *TDO2* expression as well as increased targets of AhR which is activated by kynerinine. A. The percentage of live cells (DAPI-) from TNBC attached and suspension cultures were measured by flow cytometry. B,C. The live cells (DAPI-) population was isolated by FACS after 48hrs of human TNBC (MDA-MB-453, BT549) and mouse mammary carcinoma (66cl4) suspension culture in compared with attached culture then RNA was extracted to determine TDO2 and. AhR targets- CYP1A1 and CYP1B1 expression by qPCR. *: $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by t-test.

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). (**50% completed).** Now that we know that we can enrich for TDO2, we will perform the mammosphere and tumorigenicity assays. We also wanted to manipulate TDO2 by pharmacologic and genetic knockdown and overexpression. In order to do in vivo experiments in an immune competent syngeneic model to study the effects of TDO2 inhibition on metastasis, we used the mouse mammary carcinoma 66CL4 cell line that two inhibitors of TDO2 significantly inhibit the amount of formyl-kynurenine produced. Although the about of intracellular tryptophan and kynurenine were not significantly reduced by 48 hours of inhibitor treatment, they did trend downwards. Knockdown of TDO2 with two different shRNAs demonstrated a significant reduction in TD02 gene expression that resulted in significantly less formly-kynurenine with at least one of the shRNA (shTDO2-337) (Figure 6). These results are being repeated and we will test longer time courses.



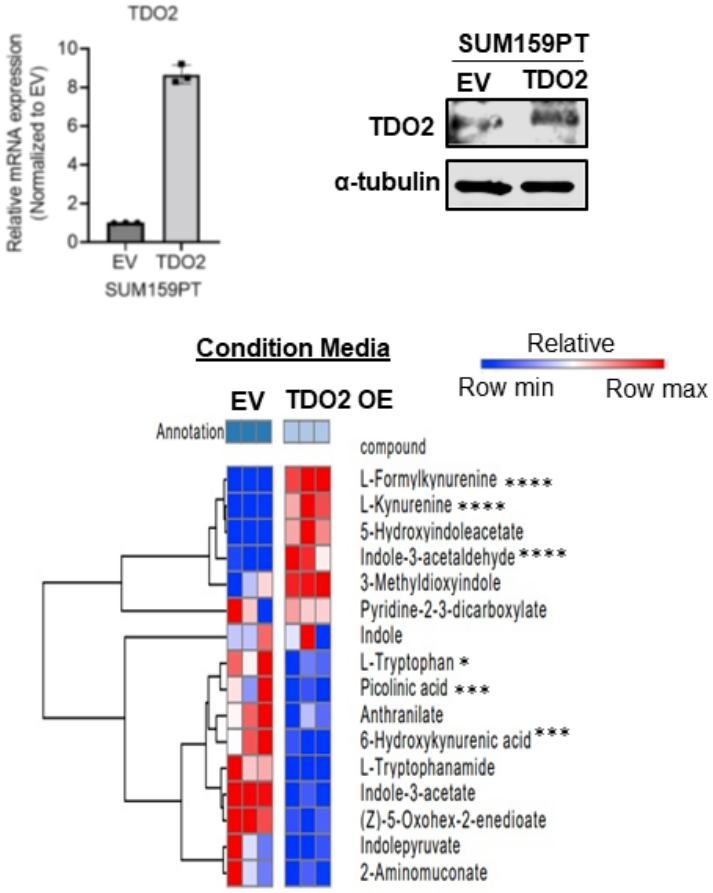


Figure 6. Pharmacological and genetic knockdown TDO2 of 66cl4 mouse mammary carcinoma decrease the TRP catabolism. *A. 66cl4 cells were treated with AT0174 for 48hrs and the cell pellets were collected for the mass spectrum analysis for the intracellular tryptophan catabolites. B. Stable silencing TDO2 by shRNA in 66cl4 and measured the mouse TDO2 mRNA expression. C. Condition media were collected from 66cl4 cells with genetic TDO2 knockdown and determined the secreted formyl-KYN and KYN. With One-way ANOVA defined *: p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

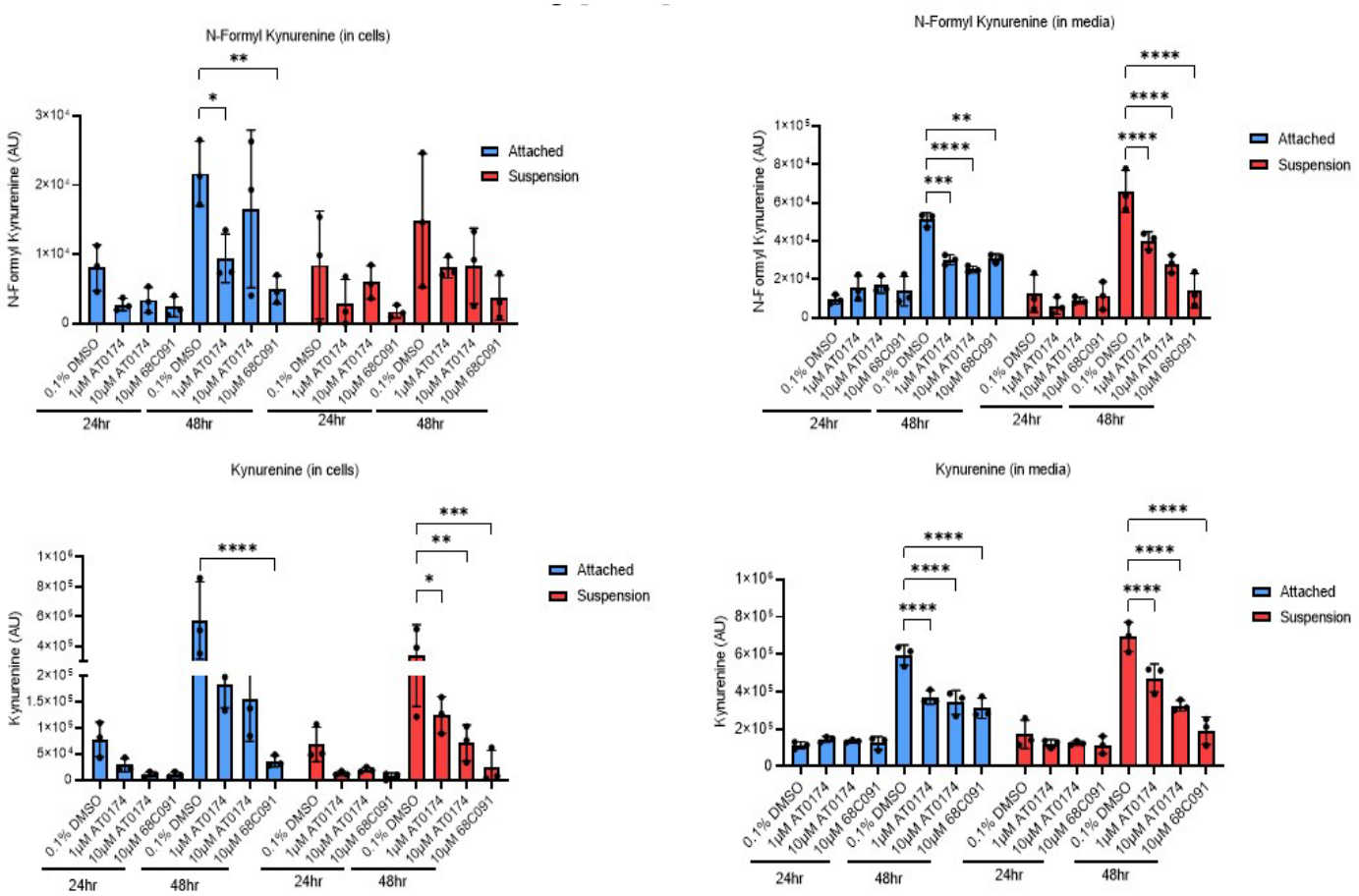
We are also performing overexpression of TDO2 in both the mouse and human models, but so far have only made the stably overexpressing human TNBC breast cancer cell line SUM159PT TDO2. Mass spectrometry demonstrated an increase in kynurenine and other tryptophan catabolites in the condition media from these cells, while tryptophan is depleted from the media (Figure 7).

Figure 7. Stable TDO2 overexpression (OE) TNBC line demonstrates the increase of tryptophan catabolism pathway activity. *A. TDO2 mRNA and protein expression determined by qPCR or immunoblot. B. Condition media (CM) were collected and analyzed by mass spectroscopy for the tryptophan downstream catabolites panel with biological replicates. The data were presented as heatmap and analyzed with t-test in comparison to the corresponding control (Empty Vector group) *: p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

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 8**).

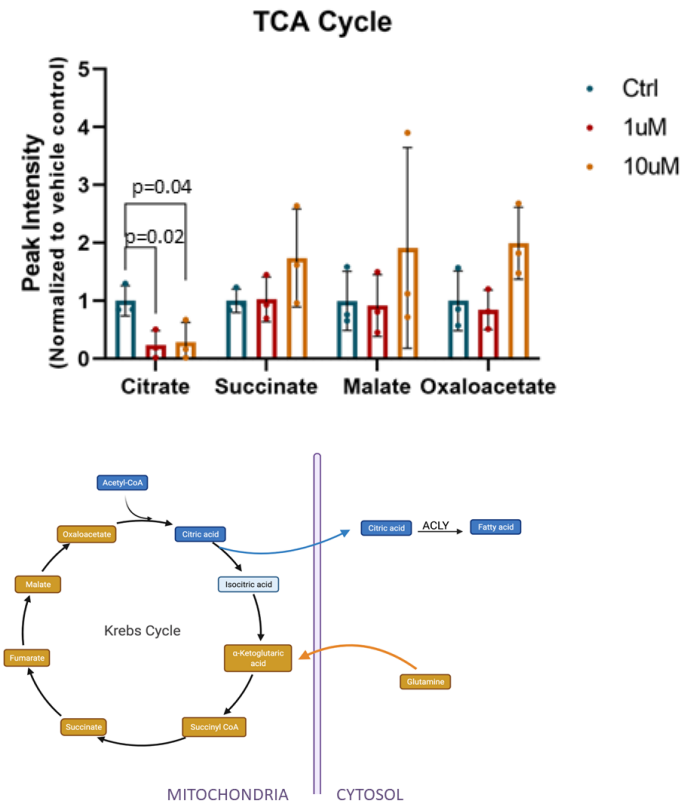
Figure 8. Two different TDO2 inhibitors decreased breast cancer production of tryptophan catabolites in a dose dependent manner both in the attached and anchorage independent (suspended) condition as determined by mass spectroscopy. *Both TDO2 inhibitors, AT0174 and 68c091 (tenatoprazole), were tested 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).*

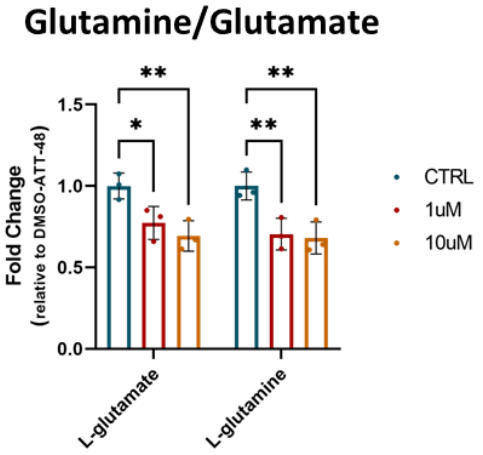


We also find that the Antido TDO2-IDO inhibitor drug AT0174 decreases citrate in the TCA cycle in a dose dependent manner. However, the later steps of the cycle generating succinate, malate and oxaloacetate were not significantly altered (**Figure 9**). One explanation for this is that citric acid is 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 9 bottom**) summarizes the conversion of glutamine through the TCA into citrate that may be used to make fatty acids. Since inhibition of TDO2/IDO by AT0174 or 680c91 reduced the intracellular glutamine, we tested

whether depleting glutamine with TDO2/IDO inhibition or using a glutaminase inhibitor with TDO2/IDO inhibition would reduce TNBC viability. **Figure 10** shows that either combination did reduce TNBC viability more significantly than either alone. We will now do a **soft agar** assay (where the cells are most reliant on TDO2 activity) and do both drugs at multiple concentrations to determine if the TDO2/IDOi and glutaminase inhibitor combination gives a truly synergistic killing of the tumor cells in a long-term anchorage-independent survival assay.

Figure 9. 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. However 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.*





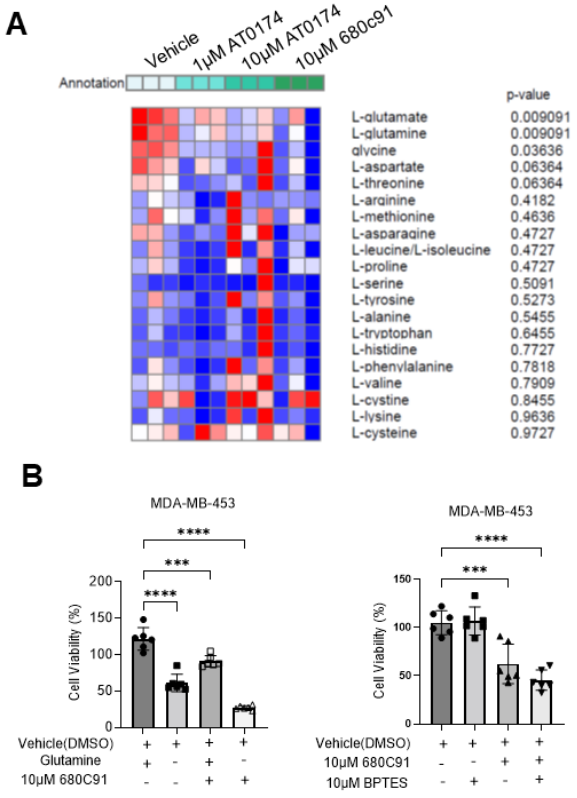


Figure 10. Inhibition of TDO2 by AT0174 or 680c91 reduces the intracellular glutamine such that combining glutamine depletion/glutaminase inhibitor with TDO2 inhibition reduces TNBC viability. *A. Global amino acid analysis shows that in MDA-MB-453 cells treated with either AT0174 or 680c91 for 24hrs cell pellets collected for mass spectrum analysis of amino acids. B. MDA-MB-453 cells were first treated with TDO2 inhibitor (680c91) for 48hrs followed by the addition of glutamine depletion (left) or the use of a glutaminase inhibitor (BPTES) (right) for 72hrs. To measure cell viability, the cells were stained with 0.1% crystal violets and measured at an absorbance 570nm. The viability was normalized to the control group (DMSO). *: p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 with One-way ANOVA.*

Task 5. Manipulate TDO2 levels and activity via direct (shRNA) and pharmacologic inhibition in syngeneic mouse mammary carcinoma model 66cl4 and measure tumor burden and TIL in primary tumor and lung metastases. (Months 12-24) (75% complete- see figure 6 above)

Task 6. Utilize the TNBC line and patient-derived xenografts (PDX) in a "humanized" mouse model to test TDO2 inhibition alone or in combination with a PD1 checkpoint inhibitor. (Months 18-30). This will be done in the upcoming year. We engineered SUM159PT, which expressed low endogenous TDO2, to overexpress TDO2 and measure the tryptophan catabolism pathway **(Figure 7 above)**. In the CM, overexpression of TDO2 increased the TRP catabolites including the secreted formyl-KYN, and KYN then lead to the depletion of TRP. Conversely, the TDO2 overexpression resulted in the decrease of the branch metabolites in the TRP catabolism pathway- picolinic acid and 6-hydrokynurenic acid. The Year 3 study will also generate the genetic knockdown of TDO2 in TNBC and transit to the humanized mouse experiments.

Milestones:

Publish manuscript on altered metabolism and tumorigenic properties associated with TDO2 75% complete. We are currently working on the manuscript draft and plan to submit it for review this fall.

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

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 presented a poster at our Cancer Biology Graduate Program Retreat in November 2021 and will present this October 2022. He will attend a Keystone meeting on tumor metabolism and metastasis in spring 2023. He is working on the manuscript that he will be co-first author on. Li-Wei just received support from our Cancer Center and the Cancer Biology T32, so he will no longer be supported by this grant, but will be working on the aims. He will attend a conference on tumor me

A postdoctoral fellow, Dr. **Lyndsey Crump**, was 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. Dr. Crump is working with Li-Wei on how tryptophan depletion and kynurenine affect not only tumor cells and T cells, but also macrophages. Lyndsey and my collaborator Dr. Ben Bitler and I are finding that tryptophan catabolism pathway is also an important mechanism of immune suppression in ovarian cancer. Dr. Crump obtained a HERA Ovarian Cancer Fund Fellowship, but it does not cover any of her salary**.** She and another postdoctoral fellow Michelle Williams are working on immune suppressive factors like both the tryptophan catabolism enzyme TDO2 and Heme Oxygenase (HO-1) that generate immune suppressive metabolites. Dr. Williams is supported now by first an NC1 F32 and now a K99.

- **How were the results disseminated to communities of interest?**

During Covid Dr. Richer gave these presentations remotely:

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"

Sept 2020 **Northwestern University Feinberg School of Medicine Department of Pharmacology**- "Targeting tryptophan catabolism in breast cancer."

- **What do you plan to do during the next reporting period to accomplish the goals?**

We will also overexpress TDO2 in the mouse model as well as knocking it down and overexpressing it in additional TNBC cell lines and patient derived xenograft models. Will compare knockdown results to pharmacologic inhibitor results to determine if the drug is hitting its target. We will then proceed to perform the proposed in vivo experiments.

4. **IMPACT:**
   - **What was the impact on the development of the principal discipline(s) of the project?**
     Beyond breast cancer, we are finding that the tryptophan catabolism pathway is also an important mechanism of immune suppression in ovarian cancer
   - **What was the impact on other disciplines?** None outside of cancer biology
   - **What was the impact on technology transfer?**
     - *Nothing to Report*
   - **What was the impact on society beyond science and technology?**
     - *Nothing to Report*
5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**

     Nothing to Report
   - **Actual or anticipated problems or delays and actions or plans to resolve them**
   - *Nothing to Report*
   - **Changes that had a significant impact on expenditures**
   - *Nothing to Report*
   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/ or select agents** *Nothing to Report*
   - **Significant changes in use or care of human subjects.** Not applicable
   - **Significant changes in use or care of vertebrate animals.** *Nothing to Report*
   - **Significant changes in use of biohazards and/or select agents**
6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
  Journal publications. *We*
    - **Books or other non-periodical, one-time publications.**
    - **Other publications, conference papers, and presentations.**
- **Website(s) or other Internet site(s)**
- **Technologies or techniques**
- **Inventions, patent applications, and/or licenses**
- **Other Products**

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

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

	Li-Wei Kuo
Project Role:	*Cancer Biology 1st year graduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	*Li-Wei performed the tryptophan tracing experiments and the experiments with the inhibitors*
Funding Support:	
	Lyndsey Crump
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	Dr. Crump is helping with the in vivo portion
	Dr. Jennifer Richer
Project Role:	supervisor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	

- **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? Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**
    - **COLLABORATIVE AWARDS:**
    - **QUAD CHARTS:**
9. **APPENDICES:** *none*