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TITLE: Targeting Tryptophan Catabolism: A Novel Method to Block Triple-Negative Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: Jennifer Richer

CONTRACTING ORGANIZATION: Regents of the University of Colorado
Aurora, CO 80045

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14. ABSTRACT Triple negative breast cancer (TNBC) cells upregulate the kynurenine pathway (KP) in forced suspension culture. The rate limiting enzyme in this pathway, TDO2 is responsible for tryptophan catabolism and production of the metabolite kynurenine (Kyn). Kyn was recently identified as an endogenous ligand for AhR, a transcription factor that was also upregulated in suspension. Kyn activation of AhR promotes motility of glioma cells. AhR is also in many immune cell types and its activation decreases T-cell activity leading to tumor immune escape. The goal of our proposal is to determine if we can target this pathway that may facilitate TNBC metastasis by enabling tumor cell invasiveness, anchorage independence and immune escape. Our hypothesis is that upregulation of kynerinine by TNBC facilitates survival in transit to metastatic sites and immune suppression and thereby mediates the highly metastatic nature of this subtype.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	11
5. Changes/Problems.....	12
6. Products.....	12
7. Participants & Other Collaborating Organizations.....	13
8. Special Reporting Requirements.....	14
9. Appendices.....	15

1. **INTRODUCTION:** Triple negative breast cancer (TNBC) cells strongly upregulate two related pathways, the kynurenine pathway (KP) in forced suspension culture. The KP is the principal route of tryptophan catabolism. Interestingly, the intermediate tryptophan metabolite kynurenine (Kyn) was recently identified as an endogenous ligand for AhR, a transcription factor that was also upregulated in suspension. Kyn activation of AhR promotes motility of glioma cells, and increased AhR expression and activity was found to be a characteristic of human and mouse mammary tumors. AhR is also in many immune cell types and its activation decreases T-cell activity leading to tumor immune escape. Since the rate limiting enzyme TDO2 increases in TNBC, the goal of our proposal is to determine if we can target this pathway that helps TNBC metastasize. Our hypothesis is that the ability to upregulate kynurenine pathway (KP) facilitates TNBC survival in suspension and mediates the highly metastatic nature of this subtype, and further, since AhR is expressed in both TNBC cells and immune cells such as effector T cells, Kyn binding to AhR can potentially elicit both autocrine effects on tumor cells themselves as well as paracrine immune-suppressive effects. To test our hypothesis we have designed the following **specific aims**: **Aim 1.** Determine if inhibition of TDO affects TNBC cell invasion, and growth on soft agar in an AhR dependent manner. **Aim 2.** Test whether inhibition of TDO-mediated kynurenine production affects the ability of TNBC to survive in the bloodstream or metastasize *in vivo* from the orthotopic site. **Aim 3.** Since immune cells such as effector T cells express AhR, we will investigate the paracrine immune-modulatory effect of Kyn secreted from TNBC. **Impact:** TDO and AhR can be targeted by small molecules, which are non-toxic in mice and there is interest in development of TDO2 and AhR inhibitors for a number of disease indications. Therefore, this represents an attractive new opportunity for targeted therapy for TNBC patients, which may revolutionize the treatment of this aggressive subtype. Following the proof of principal pre-clinical studies in this proposal, our discovery could rapidly lead to clinical trials offering new anti-metastatic treatment options for breast cancer patients.
2. **KEYWORDS:** Triple negative breast cancer (TNBC), kynurenine, tryptophan metabolism, anoikis resistance, metastasis
3. **ACCOMPLISHMENTS: What were the major goals of the project?** Our overall goals were to test the hypotheses that the ability to upregulate kynurenine via the enzyme TDO2 supports the highly metastatic nature of TNBC by two mechanisms since the receptor for kynurenine (KYN), AhR, is expressed in both TNBC cells and effector T cells, such that Kyn binding to AhR elicits both autocrine effects helping the tumor cells survive in transit as well as paracrine immune-suppressive effects.

The major goals as stated in the approved SOW are below along with completion status.

Aim 1: Determine if inhibition of TDO2 affects TNBC cell migration, invasion, and anchorage independent growth and if these effects are mediated by kynurenine activation of AhR. (Months 1-12)

Task1. Confirm that production of Kynurenine increases in TNBC cells in suspension and that this is mediated by TDO2. Compare 3 TNBC lines (BT549, SUM-159 MDA-231) to 3 luminal lines (MCF7, ZR-75.1, T47D). (Months 1-2) Complete and summarized in first year progress report and our first publication associated with this grant (D'Amato NC and Rogers T Cancer Research et al 2015, which is provided in the **appendix**). To confirm our gene array data, we performed qRT-PCR for *TDO2* and *KYNU* in multiple breast cancer cell lines, including both luminal (ER+; MCF7 and T47D) and TNBC (ER-; MDA-231, BT549, and SUM159) lines, after 24hrs in suspension (Figure 1B and C in D'Amato NC and Rogers T Cancer Research et al 2015). In all three TNBC lines tested, *TDO2* and *KYNU* were significantly increased in suspension compared to attached culture. In the two ER+ breast cancer cell lines tested, expression of these genes trended slightly higher in suspension but this change was not significant. Western blot analysis of whole cell extracts also demonstrated an increase in TDO2 and KYNU protein in TNBC cell lines (MDA-231, BT549, and SUM159) grown in suspension for 24hrs (Figure 1D and Supplemental Figure 3A). The increase in TDO2 protein was confirmed by IHC in BT549 cells grown in suspension for 48hrs compared to cells grown in the attached condition (Figure 1E). Global metabolomic profiling of intracellular and secreted metabolites from BT549 cells grown in standard attached conditions or in forced suspension for 24 hours was also performed. Two intermediate products of the kynurenine pathway, Kyn and formylkynurenine, were the intracellular metabolites with the highest fold-change increase in suspension. Among secreted metabolites, kynurenine had the third-

highest fold-change increase (Supplemental Figure 2). Together with the gene expression data, this demonstrates that the kynurenine pathway is strongly upregulated in TNBC cells upon loss of attachment. Using HPLC to verify the metabolomic profiling data, we found that secreted Kyn levels were more than two-fold higher in conditioned media from BT549 cells in forced suspension for 48hrs than in media from the same number of cells in the attached condition (Figure 1F). Furthermore, addition of the TDO2-specific inhibitor 680C91 to cells in suspension completely prevented the increase in secreted Kyn, demonstrating that increased secretion of Kyn in TNBC cells in suspension is dependent on TDO2 activity (Figure 1F).

Task 2. Determine if TDO2 or AhR knockdown or inhibition decreases migration (Months 1-4) 100% completed and summarized in first year progress report and (D'Amato NC and Rogers T Cancer Research et al 2015) in figures specified. Pharmacological inhibition of TDO2 and AhR each significantly reduced migration of MDA-MB-231 and BT549 cells in a scratch wound assay, and again the combination was more effective than either inhibitor alone (Figure 4B D'Amato NC and Rogers T Cancer Research et al 2015). Knockdown of either TDO2 or AhR recapitulated this effect, significantly diminishing migration in the scratch wound assay in both BT549 and MDA-231 cells (Figure 4C and 4D). We have yet to determine if overexpression of TDO2 increases cell migration in vitro, but we have acquired a TDO2 overexpression vector that can be stably transduced into breast cancer cell lines (TNBC, luminal (ER+), immortalized normal mammary epithelial cells like the MCF-10A) and selected using puromycin.

Task 3. Determine if TDO2 or AhR knockdown or inhibition decreases invasion (Months 5-8) 100% completed and summarized here and (D'Amato NC and Rogers T Cancer Research et al 2015). To test whether the increased TDO2 and AhR expression observed in cells grown in suspension affects invasive capacity, SUM159PT cells were grown for 48hrs either in the attached condition, or in forced suspension culture with or without addition of 10 μ M of the TDO2 inhibitor 680C91 or the AhR inhibitor CH-223191. After 24hrs, 25,000 viable cells were plated in a Matrigel-coated trans-well invasion chamber with continuous treatment. Cells grown in suspension for 24hrs were significantly more invasive than control cells grown in the attached condition (Figure 4E). The addition of the TDO2 inhibitor 680C91 during suspension culture greatly decreased the invasive capacity of viable cells. While the cells treated with the AhR inhibitor showed decreased invasion, this effect was not statistically significant (Figure 4E). We have yet to determine if overexpression of TDO2 increases cell invasion in vitro, but we have acquired a TDO2 overexpression vector that can be stably transduced into breast cancer cell lines (TNBC, luminal (ER+), immortalized normal mammary epithelial cells like the MCF-10A) and selected using puromycin.

Task 4. Test if inhibition or knockdown of TDO2 or AhR decrease anchorage-independent growth of TNBC cells in vitro (Months 9-12) Completed and summarized in first year progress report and (D'Amato NC and Rogers T Cancer Research et al 2015). Since TDO2 and AhR were upregulated by TNBC cells in suspension, we next tested their functional importance by assessing whether pharmacological inhibition or genetic knockdown could reduce anchorage-independent growth in soft agar or increase sensitivity of TNBC cells to anoikis in forced suspension culture. BT549 and SUM159PT cells were pre-treated in the attached condition with vehicle, the TDO2 inhibitor 680C91, or the AhR antagonist CH-223191 for 24hrs. Then an equal number of cells were plated in soft agar in the continued presence of treatment. Both 680C91 and CH-223191 significantly decreased anchorage-independent growth of BT549 and SUM159PT cells in soft agar (Figure 3A D'Amato NC and Rogers T Cancer Research et al 2015). To test the effect of Kyn on anoikis resistance, we treated cells in forced suspension culture for 48hrs with Kyn, and found that this significantly decreased apoptosis as measured by cleaved caspase activity compared to vehicle control treatment (Figure 3B). We then performed knockdown of TDO2 and AhR using two shRNA constructs each, and decreased protein expression was confirmed by western blot (Figure 3C). Knockdown of either TDO2 or AhR also significantly decreased growth of BT549 cells in soft agar (Figure 3D). Knockdown of either TDO2 or AhR also significantly increased apoptosis in BT549 and MDA-MB-231 cells grown in suspension for 48hrs (Figure 3E), demonstrating that TDO2 and AhR promote survival in anchorage-independent conditions. We have yet to determine if overexpression of TDO2 increases anchorage independent in vitro, but we have acquired a TDO2 overexpression vector that can be stably transduced into breast cancer cell lines (TNBC, luminal (ER+), immortalized normal mammary epithelial cells like the MCF-10A) and selected using puromycin.

Aim 2: Test whether inhibition of TDO2 and AhR affects the ability of TNBC to survive in the bloodstream following tail vein injection or to metastasize in vivo from the orthotopic site.

Task 1. Determine if TDO2 activity is critical for short term survival of TNBC cells in the vasculature (testing anoikis resistance in vivo) (Months 13-16) Complete and shown in Figure 6 of (D'Amato NC and Rogers T Cancer Research et al 2015). To test the potential contribution of TDO2 activity to the metastatic capacity of TNBC cells in vivo, we grew luciferase-expressing MDA-MB-231 cells in forced suspension conditions for 48hrs in the presence of either vehicle control or the TDO2 inhibitor 680C91. 250,000 viable cells, as determined by trypan blue staining, were then injected into the tail vein of NOD/SCID mice and luminescence was monitored over time. Seven days after injection, mice that received vehicle-treated cells had significantly higher luminescence, and this statistically significant difference was maintained throughout the experiment (Figure 6A-B D'Amato NC and Rogers T Cancer Research et al 2015). At the conclusion of the experiment at Day 28 post-injection, lung luminescence was measured ex-vivo, and lungs from mice that received vehicle-treated cells had significantly higher luminescence compared to mice that received cells treated with the TDO2 inhibitor (Figure 6C). A significant decrease in the number of metastatic nodules in the lungs from mice receiving cells treated with 680C91 was also observed by H&E (Figure 6D). Together, these data demonstrate that TDO2 inhibition decreases the ability of TNBC cells to successfully metastasize following tail vein injection in vivo.

Task. 2. Determine if pharmacological inhibition of TDO2 decreases metastasis following tail vein injection (Months 17-20) Completed and shown in Figure 6 of (D'Amato NC and Rogers T Cancer Research et al 2015).

Task 3. Determine if treatment with a small molecule inhibitor of TDO2 decreases metastasis of TNBC cells from an orthotopic xenograft model (Months 21-24). Not yet complete. As previously mentioned in our SOW, we planned on treating the mice systemically with 680C91 in drinking water and then observing effect on metastasis. However, the TDO2 inhibitor 680C91 has been previously shown to have poor stability *in vivo* and a new generation TDO2 inhibitor, LM10 was demonstrated to have superior stability in vivo (Pilotte L et al PNAS 2012). We have acquired LM10 from the Ludwig Institute for Cancer Research in Belgium through a materials transfer agreement and are analyzing the effectiveness of this compound in vitro in inhibiting kynurenine production before we begin the in vivo study. We are still optimizing the LM10 inhibitor to pharmacologically inhibit TDO2. However, only at the highest dose used 10 micromolar do we see a reduction in the amount of kynurenine produced by TNBC cells in vitro (**Figure 1**) even though it has worked in in vivo

models of other types of cancer (Pilotte L et al PNAS 2012). We are also knocking down TDO2 for comparison with LM10 to show that the effects are on target.

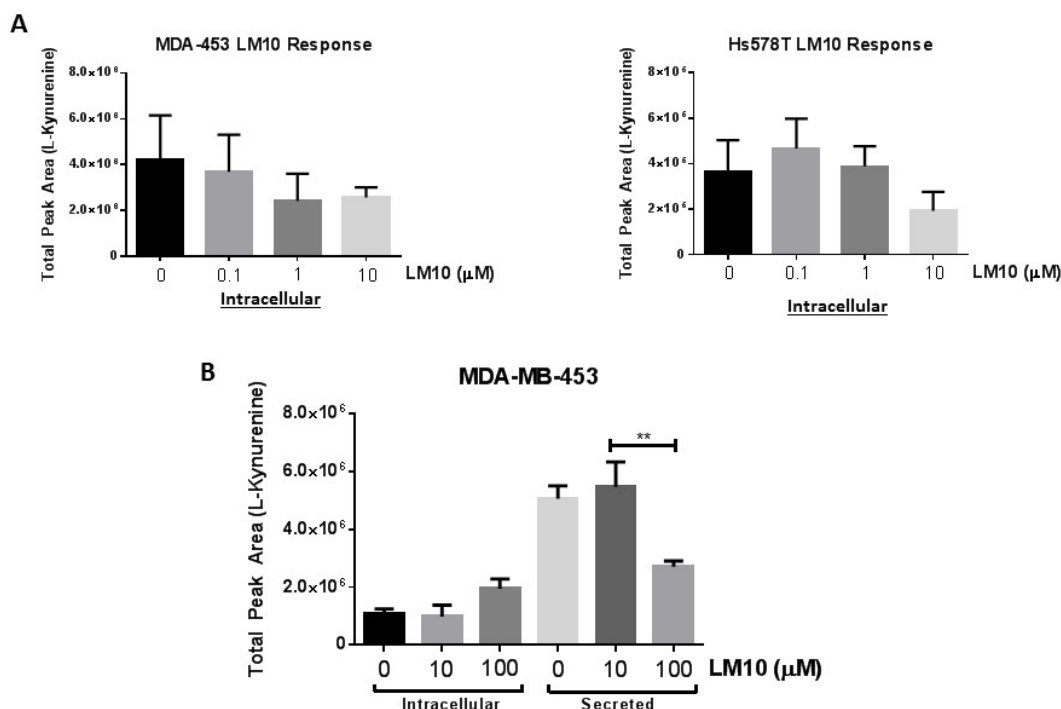


Figure 1. LM10, a second generation selective TDO2 inhibitor, alters kynurenine levels in TNBC cells. A) Relative intracellular L-kynurenine levels were measured at 72 hours in two TNBC cell lines (MDA-MB-453 and Hs578T) after treatment with various doses of LM10. B) Relative intracellular and secreted L-kynurenine levels were measured at 72 hours in MDA-MB-453 cells after treatment with various doses of LM10.

Aim 3: Identify the role of kyn paracrine action in tumor-induced immune modulation via AhR-expressing effector T cells. (Months 1-36) (100% complete). We are writing up these results now

Task 1. Determine whether conditioned media from TNBC in suspension with or without TDO inhibition alters the number and function of effector T cells via the Kyn-AhR pathway (Months 1-15) (Complete)

- a. **Isolate CD8+ and CD4+ T cells from normal donors and breast cancer patients.** CD8+ and CD4+ T-cells were both successfully isolated from the blood of healthy donors (no cancer) under the IRB-approved protocol lead by Dr. Virginia Borges using positive selection kits (Dynabeads, Invitrogen) (A pure population of CD3+ CD8+ T-cells from the blood of a healthy volunteer donors is shown in **Figure A.**). The isolation of these T-cells from the blood of breast cancer patients is currently in progress.
- b. **Activate T cells by coculture with α CD3 and α CD28 in the presence of conditioned media of TNBC cells (SUM-159 and BT549) grown in either attached condition, suspended, or suspended + 680C91.** T-cells were successfully activated over the course of 5 days using soluble anti-CD28 (1 ug/ml) and plate-bound anti-CD3 (0.5 ug/ml). The concentration of anti-CD3 and the number of T-cells plated were optimized to ensure good activation without overstimulating the T-cells (**Figure 2**).

Figure 2. Isolation of CD8+ T-cells from the blood of normal donors. Lymphocytes were isolated using Ficoll separation and then CD8+ T-cells were selected using a positive selection kit (Dynabeads, Invitrogen). Cells were then stained for CD3 and CD8 and a highly pure (95.2%) population of CD8+ T-cells was identified.

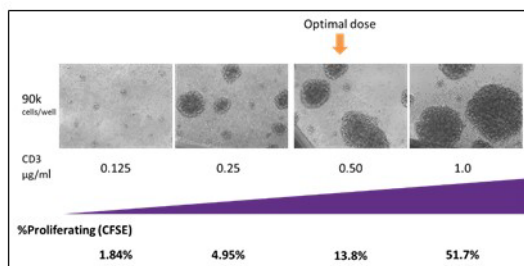
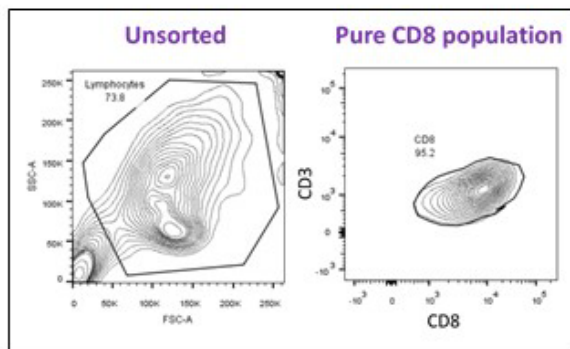
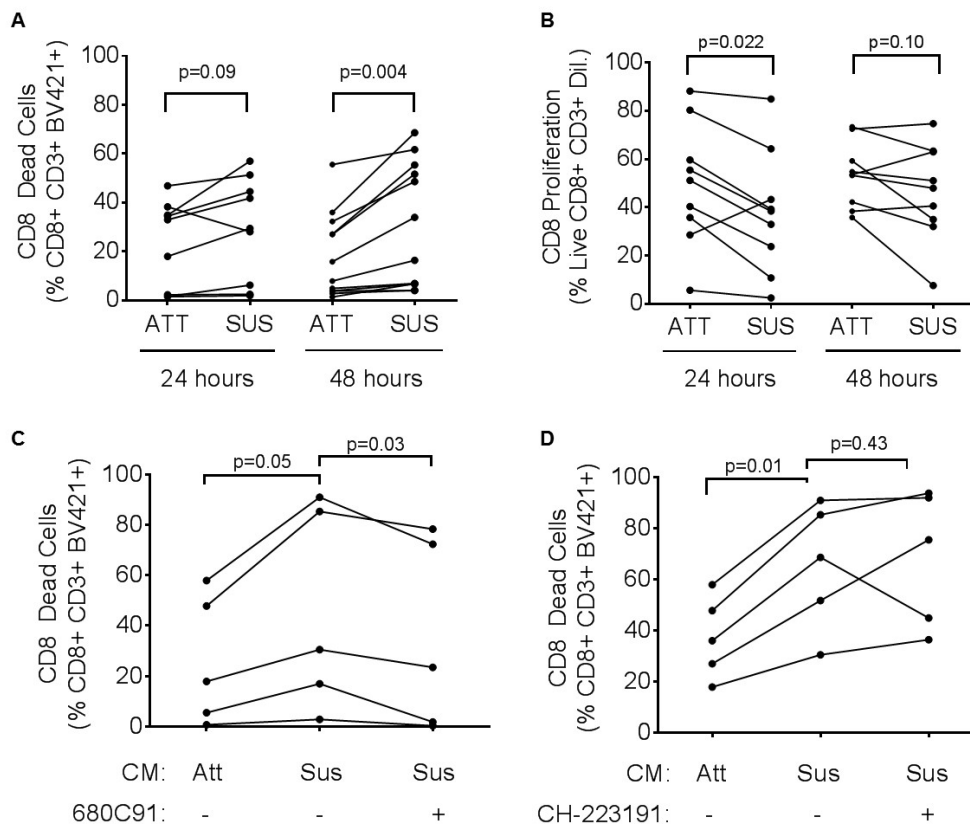


Figure 3. Optimization of T-cell activation. T-cells were isolated as described above from the blood of normal human donors, then activated over the course of 5 days using plate-bound anti-CD3 (concentrations shown) and 1 ug/ml soluble anti-CD28. Cell number was also optimized (90000 cells per well) to determine the best parameters for in vitro human T-cell activation.

- c. **Analyze T cell subsets by flow cytometry, and test proliferative capacity by CFSE, T cell viability using a fixable viability stain, and the generation of Treg cells (CD4+,FoxP3+,CD25+).** After the 5-day activation in conditioned media from TNBC cells that were grown in attached, suspended, or suspended + 680C91 conditions, T-cells were analyzed using flow cytometry. The proliferative capacity of the T-cells was measure by CFSE dilution (%diluted), and T-cell death was measured by incorporation of a fixable viability dye (eBioscience). The results of these experiments were repeated on cells from 10-12 healthy donors is in **Figure 4**.

Figure 4. The effect of conditioned media from suspended TNBC cells on primary human CD8 T-cells is similar to that of purified kynurenine and is reversed by the TDO2 inhibitor 680C91

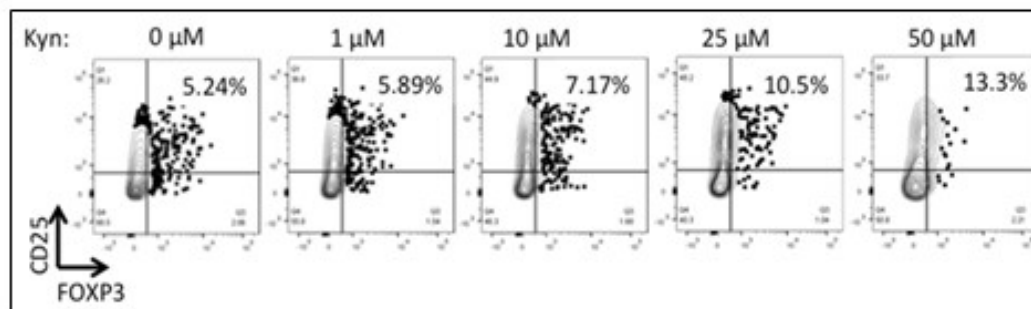
CD8 T cells were isolated from the blood of healthy human volunteer donors as described and activated for 5 days via CD3/CD28 stimulation in conditioned media from the TNBC cell line BT549, where the BT549 cells were plated in attached (ATT) or suspended (SUS) culture for either 24 or 48 hours prior to conditioned media collection. Each line represents the response of CD8 T cells from one human volunteer donor. Cell death was measured as described in Figure 1A. Paired t-tests. (B) CD8 T cell proliferation. Cells were isolated, labeled with CFSE, and cultured as described in (A). Proliferation was measured by CFSE dilution as described in Figure 1A. Paired t-tests. (C) CD8 T cell death in SUS BT549 conditioned media is reduced by TDO2 inhibition in the BT549 cells. BT549 cells were cultured in ATT or SUS conditions for 48 hours with either 0.1 μ M 680C91 (to inhibit TDO2 activity) or vehicle control (DMSO). CD8 T cells were then cultured in indicated conditioned media over a 5-day CD3/CD28 activation. Each line represents the response of CD8 T cells from one human volunteer donor. Paired t-tests. (D) CD8 T cell death in SUS BT549



conditioned media is not changed by AhR antagonism. BT549 cells were cultured in ATT or SUS conditions for 48 hours, then conditioned media was collected and CD8 T cells were activated for 5 days via CD3/CD28 in the indicated media, and treated with 10 μ M CH-223191 or vehicle control (DMSO). Paired t-tests.

- d. We found that conditioned media from attached BT549 cells increases CD8 T-cell death and decreases CD8-T-cell proliferation, indicating suppression of these T-cells. Our preliminary data also suggest that 680C91 contributes to lessening the effect of the conditioned media on the CD8 T-cells (**Figure 4C**).
- e. The impact of conditioned media on T-regs is still being investigated, but we were able to detect an increase in CD4⁺,CD25⁺,Foxp3⁺ cells in the presence of purified kynurenine (from an isolated population of CD3⁺ CD4⁺ T-cells from healthy donor blood, **Figure 5**).

Figure 5. CD4⁺ T-cells were isolated as described and then treated with purified kynurenine at the indicated doses over the course of a 5-day CD3/CD28 activation. Using flow cytometry, CD3⁺/CD4⁺ T-cells were analyzed for expression of the T-reg markers CD25 and FoxP3 (intracellular stain).



- f. **Test for functional Treg induction by coculture of stimulated CD8⁺ T cells from normal donors with CD4⁺ FoxP3⁺CD25⁺ Treg cells generated in the presence of conditioned media from TNBC cells grown in either attached condition, suspended, or suspended + 680C91.** The T-reg functionality assays are being optimized with the help of Dr. Jill Slansky's laboratory, but technique is worked out (see Figure 5).
- g. **Test in vitro for cytotoxicity function of CD8⁺ T-cells treated with conditioned media from TNBC cells grown in attached, suspended, or suspended + 680C91 (or CD8⁺ T-cells treated with Kyn) in killing tumor cells.** This has been completed and shown in Figure 4 above.

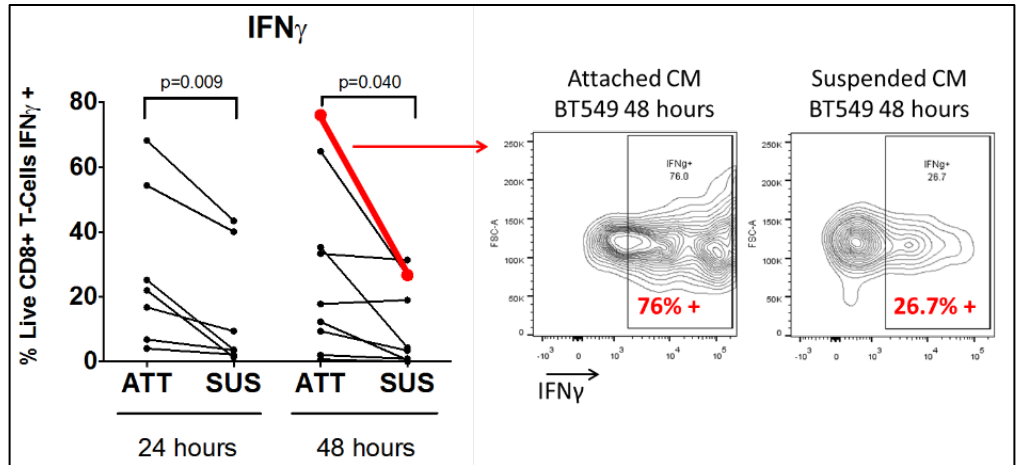
Task 2. Analyze the cytokine production of T cells stimulated with and without the TNBC-conditioned media and determine if similar cytokines are present in women with metastatic TNBC, including the AhR expression in their T cells in comparison to healthy donors (Months 5-15). (40% complete)

- h. **Isolate CD8⁺ and CD4⁺ T cells from normal donors and breast cancer patients. Completed.** See Aim 3.1.A and Figure A.
- i. **Activate T cells by coculture with αCD3 and αCD28 in the presence of conditioned media of TNBC cells grown in either attached condition, suspended, or suspended + 680C91. Completed:** See Aim 3.1.B and Figures C and D.
- j. **Analyze cytokines produced by T cells using Proinflammatory Panel 1 multiplex detection kit (MesoScale Discovery) and by using intracellular stains for interferon-gamma and granzyme-B followed by flow cytometry.** We are still optimizing the Mesoscale multiplex detection kit for this purpose, but we have used intracellular stains for interferon-gamma and granzyme B and analyzed these stains using flow cytometry. We found that conditioned media from suspended TNBC cells (BT549) does reduce interferon gamma production by CD8 T-cell (Figure 6). The impact of these conditioned media on CD4 T-cells is still being investigated.

- k. Perform western blotting and qRT-PCR to assess levels of AhR and AhR target genes such as CYP1A1 and CYP1B1 in T cells culture in different conditioned medias. These experiment are in progress.
- l. Perform western blotting using nuclear/cytosolic fractionation to assess changes in nuclear localization of AhR in T cells cultured in different conditioned medias. These experiments are in progress.

Figure 6. Conditioned media from suspended TNBC cells (BT549) reduced interferon gamma production by CD8 T-cells. CD8 T-cells were isolated as described and cultured in conditioned media from attached or suspended BT549 cells as

described over the course of a 5 day CD3/CD28 activation. Interferon gamma positivity was measured using an intracellular stain analyzed via flow cytometry. N=8 healthy human donors; paired t-test.



Task 3. Determine if metastatic potential of a syngeneic mouse breast cancer model can be altered by TDO inhibition and whether there is alteration of the tumor immune microenvironment with decreased infiltration and/or function of the tumor-infiltrating lymphocytes (Months 15-36). In planning stage. Have institutional IACUC and DOD approval. Protocol expiration date is 11/3/2019.

- m. 7×10^3 GFP-luciferase expressing 66CL4 murine breast cancer cells will be injected into the mammary fat pad of six to eight-week-old female BALB/c mice. Cells will be pretreated with 680C91 or vehicle control for 24hrs. Mice will receive either 160mg/kg/day 680C91 or LM10 or vehicle in drinking water (10 mice per group – 4 groups)
- n. Monitor luciferase expression via IVIS imaging twice per week
- o. At day 25 post-injection remove tumor, spleen, and lymph nodes
- p. Tumors will be stained by IHC to assess immune cell infiltration, cytokine expression, and TDO2 and IDO expression. Multiple immune cell types will be assessed
- q. CD4+ and CD8+ T cells from tumor tissue, spleen, and lymph nodes will be dissociated and identified, then stained for markers of activation, generation of Tregs, or exhaustion by flow cytometry

Task 4. Determine whether kynurenine effects the cytotoxic function of CD8+ T-cells *in vivo* using an *in vivo* cytotoxicity assay (Months 27-30) will be completed this year.

- r. Treat 66CL4 cells with 680C91 or LM10 or vehicle and Mitomycin C. Stain these cells and non-specific syngeneic (BALB/c background) spleen cells with different concentrations of carbofluorescein succinimidyl ester (CFSE): 66CL4 cells will be labeled with 10 uM CFSE, and splenocytes will be labeled with 1 uM CFSE
- s. Inject 2.5 million cells of each target intravenously into female BALB/C mice (10 mice per group – 2 groups)
- t. 24 hours later, isolate spleen and draining lymph nodes and isolate lymphocytes.
- u. Quantify relative numbers of cells using flow cytometry to determine the number of cells in the two peaks of CFSE staining (%CFSE-low cells)/(%CFSE-high cells)

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

Two graduate students, Thomas Rogers and Lisa Greene have been working on this project.

Thomas Rogers, Cancer Biology Doctoral Candidate 5th yr, had already obtained a F31 NRSA as reported in the last progress report, but the NCI had a new mechanism, the F99/K00 Predoctoral to Postdoctoral Fellow Transition Award (F99/K00) and Thomas was the student selected from a competitive pool at our institution to submit an application for this new mechanism and he was awarded this grant: NCI 1F99CA212230-01 (NCI Predoctoral to Postdoctoral Fellow Transition Award) Title: Targeting a Kynurenine-Driven Autocrine Loop to Block Triple-Negative Breast Cancer Metastasis Dates: 09/19/2016-8/31/2018

As a result of obtaining this award, which funds his postdoctoral work after he obtains his doctorate degree focusing for his work with me, Thomas Rogers interviewed the following prestigious places for a postdoctoral position and decided to go with Dr. Ralph DeBerardinis- UT Southwestern. Dr. DeBerardinis- is very well regarded in the field of tumor metabolism. Thomas is on track to defend his dissertation at the end of June and will finish up some experiments and join Dr. DeBerardinis's lab in September.

Dr. Costas Lyssiotis- University of Michigan

Dr. Jason Locasale- Duke University

Dr. Dan Nomura- University of California-Berkeley

Dr. Ralph DeBerardinis- UT Southwestern

Lisa Greene, Cancer Biology Doctoral Candidate 4th yr, also obtained a F31 NRSA (Ruth L. Kirschstein National Research Service Award), "Targeting TDO2 as a mediator of T-cell function in triple-negative breast cancer." NIH NCI 1F31CA203486. 2016-2019. Dates: 03/07/2016-3/07/2019

How were the results disseminated to communities of interest?

In June 2016 Dr. Richer gave a lecture at the **Lankenau Institute for Medical Research, Philadelphia, PA** guest of LIMR Director George Prendergast, Ph.D. who works on IDO in cancer, entitled "Kynurenine Pathway and TDO2 in Triple Negative Breast Cancer"

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

This coming year we will complete Aim 3 Tasks 2-4. Determine if treatment with a small molecule inhibitor of TDO2 decreases metastasis of TNBC cells from an orthotopic xenograft model (**Months 21-24**). As mentioned above, we will test a 2nd generation TDO2 inhibitor (LM10) that has shown promise in vivo in another study with a different type of cancer. Since we are just going into month 13 we will first be characterizing this compound in vitro before treating mice.

We will also be conducting Task Aim 3 to Determine if metastatic potential of a syngeneic mouse breast cancer model can be altered by TDO inhibition and whether there is alteration of the tumor immune microenvironment with decreased infiltration and/or function of the tumor-infiltrating lymphocytes (**Months 15-36**).

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project? "**
- This research is strongly suggesting that TDO2 is likely the primary enzyme that catabolizes tryptophan that should be targeted in breast cancer and that by targeting this enzyme we may be able to reduce breast cancer metastasis by decreasing the tumor cell's ability of the cells to survive in suspension, but also reverse their ability to suppress the immune system.
- **What was the impact on other disciplines?** Nothing to Report.
- **What was the impact on technology transfer?**
 - This project is likely to make an on commercial technology because it shows evidence that TDO2 is likely the primary enzyme that catabolizes tryptophan that should be targeted in breast cancer. The current drugs that are in clinical trials target IDO, not TDO2.

- **What was the impact on society beyond science and technology?** Gave two trainees great opportunities to present their work and further their career development.
- 5. **CHANGES/PROBLEMS: e:**
 - **Changes in approach and reasons for change** *None*
 - **Actual or anticipated problems or delays and actions** *None*
 - **Changes that had a significant impact on expenditures** *None*
 - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
 - *Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates. Approval of IACUC protocol by ACURO was received on 11/16/2016*
 - **Significant changes in use or care of human subjects.** None. HRPO Concurrence With the Determination of Research Not Involving Human Subjects for the Proposal, "Targeting Tryptophan Catabolism: A Novel Method to Block Triple-Negative Breast Cancer Metastasis," Submitted by Jennifer K. Richer, PhD, University of Colorado, Denver, Colorado, Proposal Log Number BC140620, Award Number W81XWH-15-1-0039, HRPO Log Number A-18613 was obtained 11/30/2016.
 - **Significant changes in use or care of vertebrate animals.** None
 - **Significant changes in use of biohazards and/or select agents.** None
- 6. **PRODUCTS:**
 - **Publications, conference papers, and presentations**

Journal publications:

1. D'Amato NC*, **Rogers TJ***, Gordon MA, Greene LI, Cochrane DR, Spoelstra NS, Nemkov TG, D'Alessandro A, Hansen KC, Richer JK. A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer. Cancer Res. 2015 Nov 1;75(21):4651-64. PubMed PMID: [26363006](#); PubMed Central PMCID: [PMC4631670](#). DOD support was acknowledged.

We are working on two additional publications now, one showing more about the regulation of TDO2 and the other how the increase in TDO2 and the resultant decrease in tryptophan and increase in kynurenine affects immune cells.

Conference presentations and posters:

Thomas also attended the 2017 Keystone Symposium- Tumor Metabolism: Mechanisms and Target meeting and gave a poster below. The abstract is included in the appendix.

Rogers TJ, Christenson JL and Richer JK. Loss of miR-200c Regulates Tryptophan Catabolism in Triple-Negative Breast Cancer. Keystone Symposium- Tumor Metabolism: Mechanisms and Targets; 2017 January; Whistler, BC "*Endogenous activation of the aryl hydrocarbon receptor promotes triple-negative breast cancer metastasis*".

Lisa Greene has also submitted an abstract to the Gordon Conference on the Mammary Gland and Breast Cancer to be held June 2107. The abstract is included in the appendix.

Website(s) or other Internet site(s)

None at this time.

Technologies or techniques

None at this time.

Inventions, patent applications, and/or licenses

None at this time.

Other Products

Identify any other reportable outcomes that were developed under this project. Examples include:

- biospecimen collections: FFPE blocks of lungs with metastases of MDA-MB-231 xenografts of tumors pretreated with or without TDO2 inhibitor
- research material: knowledge of PDX that express TDO2 or AhR.]
-

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?

RICHER,JENNIFER									
63010030 -- DOD TRYPTOPHAN CATA	14,189.48	3,636.48	17,825.96	9.38%	16,574.34	4,227.31	20,801.65	9.75%	
BORAKOVE,MICHELLE MARIE									
63010030 -- DOD TRYPTOPHAN CATA	8,563.98	3,950.01	12,513.99	15.09%	8,997.30	3,945.36	12,942.66	15.09%	
SLANSKY PH.D.,JILL E									
63010030 -- DOD TRYPTOPHAN CATA	6,385.52	1,707.20	8,092.72	5.00%	6,625.02	1,680.80	8,305.82	5.00%	
SPOELSTRA,NICOLE S									
63010030 -- DOD TRYPTOPHAN CATA	11,917.49	3,642.45	15,559.94	23.16%	18,642.87	5,584.22	24,227.09	35.32%	
CARSON BUTTERFIELD,KIEL									
63010030 -- DOD TRYPTOPHAN CATA	18,167.30	6,564.95	24,732.25	50.00%	10,056.61	3,469.33	13,525.94	25.54%	
MOORE,BRANDON LEE									
63010030 -- DOD TRYPTOPHAN CATA	383.34	196.03	579.37	3.71%	0.00	0.00	0.00	0.00	
BRUNO,TULLIA CARMELA									
63010030 -- DOD TRYPTOPHAN CATA	846.72	198.16	1,044.88	3.38%	0.00	0.00	0.00	0.00	
GREENE,LISA IRIS									
63010030 -- DOD TRYPTOPHAN CATA	19,524.36	19.52	19,543.88	72.33%	11,250.00	(19.52)	11,230.48	37.90%	
ROGERS,THOMAS									
63010030 -- DOD TRYPTOPHAN CATA	14,062.50	10.28	14,072.78	45.18%	0.00	0.00	0.00	0.00	
D'ALESSANDRO,ANGELO									
63010030 -- DOD TRYPTOPHAN CATA	8,857.18	1,675.07	10,532.25	9.69%	8,857.00	2,143.29	11,000.29	6.96%	

○

Researcher Identifier CAGE code is OP6C1 for all

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
 - No
- **What other organizations were involved as partners?**
 - *No*

8. SPECIAL REPORTING REQUIREMENTS N/A

9. APPENDIX

D'Amato NC*, **Rogers TJ***, Gordon MA, Greene LI, Cochrane DR, Spoelstra NS, Nemkov TG, D'Alessandro A, Hansen KC, Richer JK. A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer. Cancer Res. 2015 Nov 1;75(21):4651-64. PubMed PMID: [26363006](#); PubMed Central PMCID: [PMC4631670](#).

Two abstracts:

Lisa Greene has also submitted an abstract to the Gordon Conference on the Mammary Gland and Breast Cancer to be held June 2107

Thomas Rogers Keystone Symposium- Tumor Metabolism: Mechanisms and Targets; 2017 January;

A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer

Nicholas C. D'Amato¹, Thomas J. Rogers¹, Michael A. Gordon¹, Lisa I. Greene¹, Dawn R. Cochrane¹, Nicole S. Spoelstra¹, Travis G. Nemkov², Angelo D'Alessandro², Kirk C. Hansen², and Jennifer K. Richer¹

Abstract

The ability of a cancer cell to develop resistance to anoikis, a programmed cell death process triggered by substratum detachment, is a critical step in the metastatic cascade. Triple-negative breast cancers (TNBC) exhibit higher rates of metastasis after diagnosis, relative to estrogen-positive breast cancers, but while TNBC cells are relatively more resistant to anoikis, the mechanisms involved are unclear. Through gene expression and metabolomic profiling of TNBC cells in forced suspension culture, we identified a molecular pathway critical for anchorage-independent cell survival. TNBC cells in suspension upregulated multiple genes in the kynurenine pathway of tryptophan catabolism, including the enzyme tryptophan 2,3-dioxygenase (TDO2), in an NF- κ B-dependent manner. Kynurenine production mediated by TDO2 in TNBC cells was sufficient to activate aryl hydrocarbon

receptor (AhR), an endogenous kynurenine receptor. Notably, pharmacologic inhibition or genetic attenuation of TDO2 or AhR increased cellular sensitivity to anoikis, and also reduced proliferation, migration, and invasion of TNBC cells. *In vivo*, TDO2 inhibitor-treated TNBC cells inhibited colonization of the lung, suggesting that TDO2 enhanced metastatic capacity. In clinical specimens of TNBC, elevated expression of TDO2 was associated with increased disease grade, estrogen receptor-negative status, and shorter overall survival. Our results define an NF- κ B-regulated signaling axis that promotes anoikis resistance, suggest functional connections with inflammatory modulation by the kynurenine pathway, and highlight TDO2 as an attractive target for treatment of this aggressive breast cancer subtype. *Cancer Res*; 75(21); 4651–64. ©2015 AACR.

Introduction

The vast majority of breast cancer deaths are caused by complications from metastases (1–3). A high rate of metastasis is characteristic of triple-negative breast cancers (TNBC), which lack expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 amplification, and thus do not respond to current endocrine therapies or HER2-targeted therapies (4). Due in part to the absence of effective targeted therapies, but likely also to inherent properties of this subtype, patients with metastatic TNBC have a poor prognosis with a median survival of 13 months (5). Thus, identification of new targeted therapies that inhibit or slow metastasis is of critical importance to improve the prognosis of women with TNBC.

Metastasis is a complex process with multiple steps, including detachment of cancer cells from the primary tumor, invasion through local tissue, intravasation into the vasculature and lymphatics, survival while in transit, extravasation, and colonization of secondary sites (6). Normal epithelial cells are programmed to undergo apoptosis if they become detached from the basement membrane, a process termed anoikis (7). Anoikis resistance is thought to facilitate survival of tumor cells that detach from the primary tumor and thereby facilitate metastasis (8, 9). Multiple mechanisms of anoikis resistance have been identified, including deregulation of integrin expression and aberrant activation of prosurvival pathways (as reviewed in ref. 9), as well as altered metabolism (10, 11). However, the present study is the first to globally profile gene expression alterations in forced suspension culture with the goal of identifying targetable pathways important for survival in suspension. This objective screening approach identified multiple components of the kynurenine pathway of tryptophan catabolism and the aryl hydrocarbon receptor (AhR), which is activated by kynurenine (12), as being upregulated by TNBC in suspension culture.

Altered tryptophan metabolism and increased secretion of tryptophan metabolites by solid tumors, including in breast cancer, have long been recognized (13, 14). The essential amino acid tryptophan is required for protein synthesis and is a precursor for the formation of multiple signaling molecules including serotonin (15). The majority of tryptophan catabolism occurs via the kynurenine pathway, leading to synthesis of NAD⁺ along with intermediate products, including quinolinic acid and

¹Department of Pathology, University of Colorado Anschutz Medical Campus, Aurora, Colorado. ²Department of Biochemistry and Molecular Genetics, University of Colorado Anschutz Medical Campus, Aurora, Colorado.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

N.C. D'Amato and T.J. Rogers contributed equally to this article.

Corresponding Author: Jennifer K. Richer, University of Colorado at Denver, RCI North P18-5127 Mail Stop 8104, 12800 E. 19th Ave, Aurora, CO 80045. Phone: 303-724-3735; Fax: 303-724-3712; E-mail: Jennifer.Richer@ucdenver.edu

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D'Amato et al.

kynurenine (Kyn; ref. 16). The first step of the kynurenine pathway can be catalyzed by indoleamine 2,3-dioxygenase 1 (IDO1), IDO2, or tryptophan 2,3-dioxygenase (TDO2; refs. 17–19). IDO1 is expressed in tissues throughout the body, whereas TDO2 is predominantly expressed in the liver (20). Much research on the kynurenine pathway in cancer has focused on the ability of Kyn to decrease immune surveillance (as reviewed in ref. 21), but in glioma, Kyn, acting in both a paracrine and an autocrine fashion, suppressed antitumor immune responses and promoted tumor cell survival and motility. Kyn was demonstrated to serve as an endogenous ligand to activate AhR in both immune cells and the tumor cells themselves (12).

AhR is a member of the basic-helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) superfamily of transcription factors best known for its activation by xenobiotics such as polycyclic aromatic hydrocarbons (PAH; refs. 22, 23). PAH binding leads to transcription of AhR target genes, including drug-metabolizing enzymes such as *CYP1A1* and *CYP1B1* (24). Increased expression of AhR and its target genes has been found in several cancer types, including lung, cervical, ovarian, and breast (16). AhR is required for normal mammary gland development (25, 26), and AhR overexpression increased migration, invasion, and proliferation of immortalized mammary epithelial cells (27). AhR knockdown in MDA-MB-231 TNBC cells decreased expression of genes involved in these same processes (28). Interestingly, increased AhR activity in mouse hepatoma cells grown in suspension was observed nearly 20 years ago (29); however, neither the mechanism of activation nor the functional significance was tested.

Using global expression analysis and metabolomic profiling of TNBC cells in forced suspension, we identify a novel TDO2-AhR signaling axis, mechanistically dependent on NF- κ B, which promotes anoikis resistance, as well as migratory and invasive capacity. Indeed, we found that pharmacologic inhibition or knockdown of TDO2 or AhR decreased anchorage-independent growth and invasive capacity *in vitro* and TDO2 inhibition decreased lung metastasis in a TNBC preclinical model. Collectively, these data and the fact that *TDO2* expression confers a shorter overall survival in breast cancer patients suggest that TDO2 inhibition may be a rational targeted therapy to reduce TNBC metastasis and resultant mortality.

Materials and Methods

Cell culture and treatments

All cell lines were authenticated by short tandem repeat analysis and tested negative for mycoplasma in July of 2014. SUM159PT cells were purchased from the University of Colorado Cancer Center Tissue Culture Core in August of 2013 and were grown in Ham's F-12 with 5% FBS, penicillin/streptomycin, hydrocortisone, insulin, HEPES, and L-glutamine supplementation. MDA-MB-231 (MDA-231) cells were purchased from the ATCC in August of 2008 and were grown in minimum essential media with 5% FBS, penicillin/streptomycin, HEPES, L-glutamine, non-essential amino acids, and insulin supplementation. BT549 cells, purchased from the ATCC in 2008, were grown in RPMI Medium 1640 with 10% FBS, penicillin/streptomycin, and insulin. MCF7 and T47D cells were purchased from the ATCC and were grown in DMEM with 10% FBS and 2 mmol/L L-glutamine.

Forced suspension culture

Poly-2-hydroxyethyl methacrylate (poly-HEMA, from Sigma-Aldrich) was reconstituted in 95% ethanol to 12 mg/mL. Ethanol

was allowed to evaporate overnight, and plates were washed with PBS prior to use.

Kynurenine high performance liquid chromatography

Experimental samples were generated by plating 500,000 cells in 2 mL culture media in a 6-well plate in triplicate. After 48 hours, media were collected and protein was precipitated using trichloroacetic acid at a final concentration of 7%, samples were centrifuged, and the supernatant was analyzed on an Agilent 1260 high performance liquid chromatography (HPLC) with a Kinetex BiPhenyl column (particle size 2.7 μ m, 4 mm internal diameter by 150 mm; Phenomenex, catalog no. 00F-4622-E0). Kynurenine was eluted using a binary gradient consisting of 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in acetonitrile (mobile phase B). The separation gradient was 5% to 60% B over 6 minutes at a flow rate of 0.75 mL/min, and the kynurenine peak was monitored at 360 nm. To quantify kynurenine in the samples, peak areas were compared with a serial dilution of L-kynurenine (Sigma; catalog no. K8625) in tissue culture media, which was prepared with the same precipitation method as the samples. In brief, 100 μ L of standard or supernatant was injected onto the system, and peak area was calculated using Agilent ChemStation software.

Cellular assays and reagents

Cells were treated with 680C91 and CH223191 (TOCRIS Bioscience) prepared in dimethyl sulfoxide (DMSO). Alpha-naphthoflavone (Sigma-Aldrich) was diluted in methanol:ethyl acetate (3:1), and 1-D,L-methyl-tryptophan and crystalline L-kynurenine (Sigma-Aldrich) were prepared in 0.25N HCl. PS 1145 (TOCRIS Bioscience) was prepared in DMSO. IL1 β and TNF α were obtained from eBioscience.

Cellular fractionation

Cellular fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology) as per the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Plus Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized with the Applied Biosystems reverse transcription Kit. qRT-PCR was performed in an ABI 7600 FAST thermal cycler using Absolute Blue qPCR SYBR Green Low ROX Mix (Thermo Scientific). All fold-change data were normalized to β -actin. All experiments were performed in biologic triplicate. For primer sequences, see Supplementary Methods.

Immunoblotting

Whole-cell protein extracts (50 μ g) were denatured, separated on SDS-PAGE gels, and transferred to polyvinylidene fluoride membranes. After blocking in 3% BSA in Tris-buffered saline-Tween, membranes were probed overnight at 4°C. Primary antibodies used include AhR (#13790; 1:1,000 dilution; Cell Signaling Technology), KYN (H00008942-B01; 1:1,000 dilution; NOVUS Biologicals), TDO2 (H00006999-B01P; 1:1,000 dilution; Abnova), TOPO1 (C-21; 1:100 dilution; Santa Cruz Biotechnology, Inc.), and α -tubulin (clone B-5-1-2; 1:30,000 dilution; Sigma Aldrich). Following secondary antibody incubation, results were detected using Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer). Densitometry quantification was performed using Image Studio Lite

Version 3.1 and reported as a ratio compared with α -tubulin as a loading control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue sections (5 μ m) were heat immobilized onto glass slides and deparaffinized in a series of xylenes and graded ethanols. Antigens were heat retrieved in 10 mmol/L Tris, 1 mmol/L EDTA, pH 9.0 solution, and blocked for endogenous peroxidase followed by 10% normal goat serum prior to antibody incubation. Tris-buffered saline with 0.05% Tween (TBST) was used for all washes. TDO2 antibody (Abnova; # H00006999-B01P) was incubated overnight at room temperature at 1:200 in TBST, and AhR antibody (CST #13790) was incubated for 1 hour at room temperature at 1:50 in TBST. Envision polymer (Dako) was used for detection, followed by 3,3'-diaminobenzidine (Dako), and slides were counterstained with dilute hematoxylin.

Gene expression array analysis

BT549 cells were grown in either attached conditions or forced-suspension conditions on poly-HEMA-coated plates in quadruplicate for 24 hours. RNA was harvested at 24 hours using Trizol method, and hybridized onto Affymetrix Human Gene 1.0ST arrays at the University of Colorado Denver Genomics and Microarray Core, following the manufacturer's instruction.

Microarray analysis was performed using Partek Genomics Suite (Partek, Inc.) and Ingenuity Pathway Analysis software (Qiagen, Inc.). One-way ANOVA analysis was performed to determine differentially expressed genes between the two treatment groups (attached vs. suspended). Fold-change cutoff was 1.5, and significance cutoff was $P < 0.05$. Significantly differentially expressed genes were imported to Ingenuity for pathway analysis, including identification of altered canonical pathways.

shRNA experiments

High-titer shRNA lentiviral transduction particle suspensions were obtained from the Functional Genomics Facility at the University of Colorado (Boulder, CO). BT549 or MDA-MB-231 cells were plated at 50% confluence in 60-mm tissue culture-treated dishes in 4 mL of media. Twenty-four hours after plating, 100 μ L of the lentiviral suspension and 8 μ g/mL polybrene (Sigma-Aldrich) were added to the tissue culture media. After 24 hours, viral media were replaced with regular tissue culture media and cells were incubated for an additional 24 hours before the commencement of puromycin selection (1 μ g/mL). Knock-downs were confirmed by Western blot, and all experiments were done within three passages of puromycin selection. For shRNA sequences, see Supplementary Methods.

Luciferase reporter activity

The AhR luciferase reporter, generously provided by Dr. Michael Denison (University of California-Davis), and NF- κ B luciferase reporter, generously provided by Dr. Rebecca Schweppe (University of Colorado-Anschutz Medical Campus), were transiently transfected along with SV40 Renilla in TNBC cells using Lipofectamine (Life Technologies). Transfected cells were then plated in a 24-well plate at a density of 5×10^5 cells per well in either a control or poly-HEMA-coated well and incubated at 37°C for 24 hours. Following incubation, reporter activation was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Briefly, cells

were lysed for 15 minutes at room temperature using $1 \times$ passive lysis buffer. Lysed cells were collected and centrifuged at 14,000 rpm for 15 minutes at 4°C to eliminate cell debris. The supernatant was used immediately or diluted with $1 \times$ passive lysis buffer for determination of luciferase activity. For analysis, AhR or NF- κ B reporter activity was normalized to SV40 reporter activity to control for differences in transfection efficiency.

Measurement of anoikis

Soft-agar assays were performed in 0.5% bottom and 0.25% top-layer agar (Difco Agar Noble; BD Biosciences). Media with treatment were refreshed every 4 days.

Caspase-3/7 activity was measured using a Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's protocol. Briefly, 8×10^4 TNBC cells were plated in a clear bottom, white-walled 96-well plate coated with poly-HEMA. The cells were incubated for 48 hours at 37°C. Following incubation, each well was mixed with Caspase-Glo 3/7 reagent in equal volume to the culture medium. The 96-well plate was then covered in foil, shook for 30 seconds, and incubated at room temperature for 30 minutes. Luminescence was determined by luminometer, measuring luminescence after 1 second.

In vivo metastasis experiment

Tail-vein injection experiments were approved by the University of Colorado Institutional Animal Care and Use Committee [IACUC protocol—83612(10)1E]. All animal experiments were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. Prior to tail-vein injection, luciferase-expressing MDA-MB-231 cells were grown in forced suspension conditions for 48 hours in the presence of either vehicle control or 10 μ mol/L 680C91. A total of 250,000 viable cells, as determined by trypan blue staining, were then injected into the tail vein of NOD.CB17-Prkdc^{scid}/J (NOD/SCID) mice (The Jackson Laboratory). Following injection, luminescence was monitored every 7 days for a total of 4 weeks. At the completion of this experiment, lungs of 5 mice from each group were imaged by IVIS *ex vivo*. Lungs from the remaining 5 mice per group were formalin-fixed and paraffin-embedded for IHC.

Proliferation, migration, and invasion

Proliferation assays were performed using the Incucyte ZOOM live-cell imaging system (Essen BioSciences). Migration scratch wound assays were performed per the manufacturer's instructions and scanned with the Incucyte ZOOM apparatus (Essen BioSciences). Trans-well invasion assays were performed with BD BioCoat Matrigel Invasion Chambers (BD Biosciences) per the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. Student *t* test, ANOVA with Tukey post-hoc test, and two-way ANOVA with Bonferroni multiple comparison test were used as noted. *P* values are denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

Results

Multiple enzymes of the kynurenine pathway are upregulated in detached TNBC cell lines

To model loss of attachment *in vitro*, we grew breast cancer cells on tissue culture plates coated with poly(2-hydroxyethyl

methacrylate) (poly-HEMA). We previously demonstrated that TNBC cells grown in forced suspension upregulate proteins involved in anoikis resistance and motility (30, 31). To identify additional pathways responsible for resistance to anoikis in an unbiased, global fashion, expression profiling was performed on the TNBC cell line BT549 grown in standard attached conditions for 24 hours as compared with the same number of cells grown in forced suspension for 24 hours in quadruplicate. In total, we identified 367 genes that change more than 1.5-fold in a statistically significant manner ($P < 0.05$, log-rank test). Of these genes, 217 (59%) were upregulated and 150 (41%) were downregulated in suspended cells (Supplementary Fig. S1A). Ingenuity Pathway Analysis of these data revealed two related pathways strongly upregulated in suspension: tryptophan catabolism and AhR signaling (Supplementary Fig. S1B and S1C).

Figure 1A shows unsupervised hierarchical clustering of the top 35 genes significantly upregulated by at least 2-fold in suspended compared with attached conditions ($P < 0.05$, log-rank test). Among the genes most highly upregulated in suspension were two enzymes involved in the kynurenine pathway, which converts tryptophan to NAD^+ . The rate-limiting enzyme tryptophan 2,3-dioxygenase (*TDO2*) was the most highly upregulated gene in suspension, and the downstream enzyme kynureninase (*KYNU*) was also among the top fold-changing genes (Fig. 1A). To confirm our gene array data, we performed qRT-PCR for *TDO2* and *KYNU* in multiple breast cancer cell lines, including both luminal (ER^+) and TNBC (ER^-) lines, after 24 hours in suspension (Fig. 1B and C). In all three TNBC lines tested, *TDO2* and *KYNU* were significantly increased in suspension compared with attached culture. In the two ER^+ breast cancer cell lines tested, expression of these genes trended slightly higher in suspension, but this change was not significant.

Western blot analysis of whole-cell extracts also demonstrated an increase in *TDO2* and *KYNU* proteins in TNBC cell lines (MDA-231, BT549, and SUM159) grown in suspension for 24 hours (Fig. 1D; and Supplementary Fig. S3A). The increase in *TDO2* protein was confirmed by IHC in BT549 cells grown in suspension for 48 hours compared with cells grown in the attached condition (Fig. 1E).

Global metabolomic profiling of intracellular and secreted metabolites from BT549 cells grown in standard attached conditions or in forced suspension for 24 hours was also performed. Two intermediate products of the kynurenine pathway, Kyn and formylkynurenine, were the intracellular metabolites with the highest fold-change increase in suspension. Among secreted metabolites, kynurenine had the third-highest fold-change increase (Supplementary Fig. S2). Together with the gene expression data, this demonstrates that the kynurenine pathway is strongly upregulated in TNBC cells upon loss of attachment.

Using HPLC to verify the metabolomic profiling data, we found that secreted Kyn levels were more than 2-fold higher in conditioned media from BT549 cells in forced suspension for 48 hours than in media from the same number of cells in the attached condition (Fig. 1F). Furthermore, addition of the *TDO2*-specific inhibitor 680C91 to cells in suspension completely prevented the increase in secreted Kyn, demonstrating that increased secretion of Kyn in TNBC cells in suspension is dependent on *TDO2* activity (Fig. 1F). *TDO2* mRNA was expressed at a higher copy number at baseline than *IDO1* in two of the three TNBC cell lines tested, and *TDO2* expression increased more strongly in suspension than *IDO1* (Supplementary Fig. S3B).

Aryl hydrocarbon receptor expression and activity are increased in suspension

In addition to the components of the kynurenine pathway, *AhR* and *AhR*-regulated genes were also identified among the genes most highly upregulated by TNBC cells in suspension (Fig. 1A and Supplementary Fig. S4). Increased *AhR* mRNA expression in suspended compared with attached TNBC cells was confirmed by qRT-PCR. Similar to *TDO2* and *KYNU*, *AhR* was not significantly increased in ER^+ cell lines in suspension (Fig. 2A). Furthermore, *AhR* protein was increased in all three TNBC cell lines tested following 24 hours in suspension as tested by Western blot (Fig. 1D; Supplementary Fig. S3A), and in BT549 cells grown in suspension for 48 hours as measured by IHC (Fig. 2C). In attached SUM159 cells, while *AhR* protein was found in both the cytoplasmic and nuclear fractions, after 48 hours in forced suspension, *AhR* protein was almost exclusively nuclear (Fig. 2B). Because *AhR* nuclear translocation and transcriptional activity are known to be ligand-mediated (32), the movement of *AhR* to the nucleus in suspension suggests that the receptor is ligand-bound and transcriptionally active.

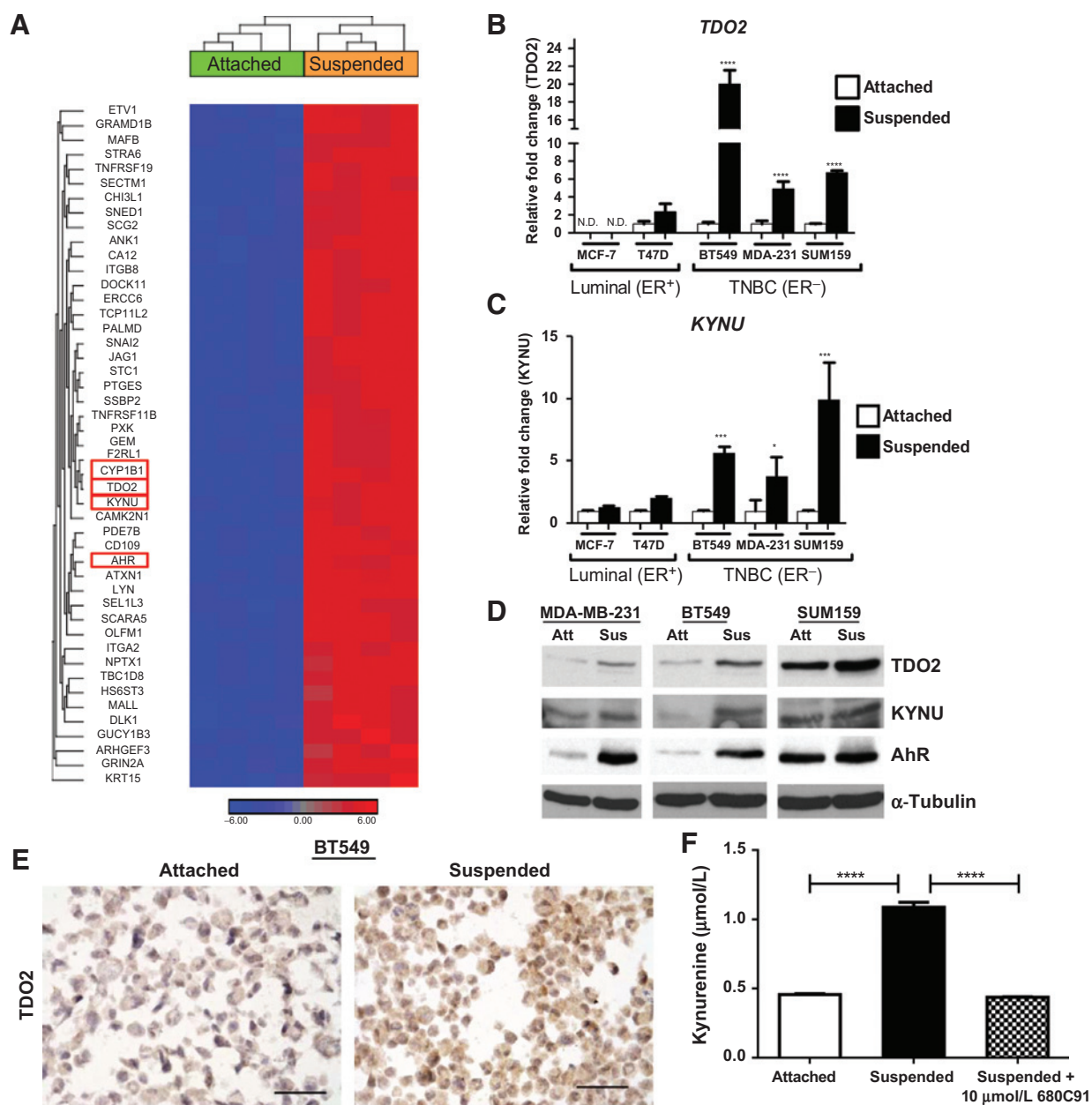
We assessed *AhR* transcriptional activity using a luciferase reporter containing six dioxin response elements (DRE), the consensus *AhR*-binding site. In BT549 and MDA-231 cells, luciferase activity was upregulated more than 2-fold in suspended compared with attached cells (Fig. 2D). The *AhR* antagonist α -naphthoflavone inhibited the suspension-mediated increase in luciferase activity, demonstrating specificity of the reporter construct for *AhR* activity (Fig. 2D; Supplementary Fig. S3C).

TDO2-mediated kynurenine production activates *AhR*

Kyn is an endogenous ligand for *AhR* (12, 33–35), suggesting a potential relationship between increased kynurenine pathway enzyme expression and increased *AhR* activity in TNBC cells in suspension. First, we tested whether inhibition of *TDO2* and consequent Kyn production could prevent *AhR* activation in suspended TNBC cells. Treatment with 10 $\mu\text{mol/L}$ 680C91, which prevented the suspension-induced increase in Kyn, significantly decreased *AhR* activity in BT549 and MDA-MB-231 cells in suspension (Fig. 2E; Supplementary Fig. S3D). Furthermore, the suspension-mediated increase in *AhR* target genes *CYP1A1* and *CYP1B1* was abrogated by both the *AhR* inhibitor CH-223191 and the *TDO2* inhibitor 680C91 (Fig. 2F), linking *TDO2* activity to *AhR* transcriptional activity. Addition of 100 $\mu\text{mol/L}$ exogenous Kyn also resulted in a significant increase in *AhR* luciferase reporter activity in TNBC cells in both attached and suspended culture conditions (Fig. 2G), demonstrating that Kyn is able to activate *AhR* in TNBC cells.

Inhibition of *TDO2* and *AhR* decreases TNBC anoikis resistance, proliferation, migration, and invasion

Because *TDO2* and *AhR* were upregulated by TNBC cells in suspension, we next tested their functional importance by assessing whether pharmacologic inhibition or genetic knockdown could reduce anchorage-independent growth in soft agar or increase sensitivity of TNBC cells to anoikis in forced suspension culture. BT549 and SUM159PT cells were pretreated in the attached condition with vehicle, the *TDO2* inhibitor 680C91, or the *AhR* antagonist CH-223191 for 24 hours. Then an equal number of cells were plated in soft agar in the continued presence of treatment. Both 680C91 and CH-223191 significantly decreased anchorage-independent growth of BT549 and SUM159 cells in soft

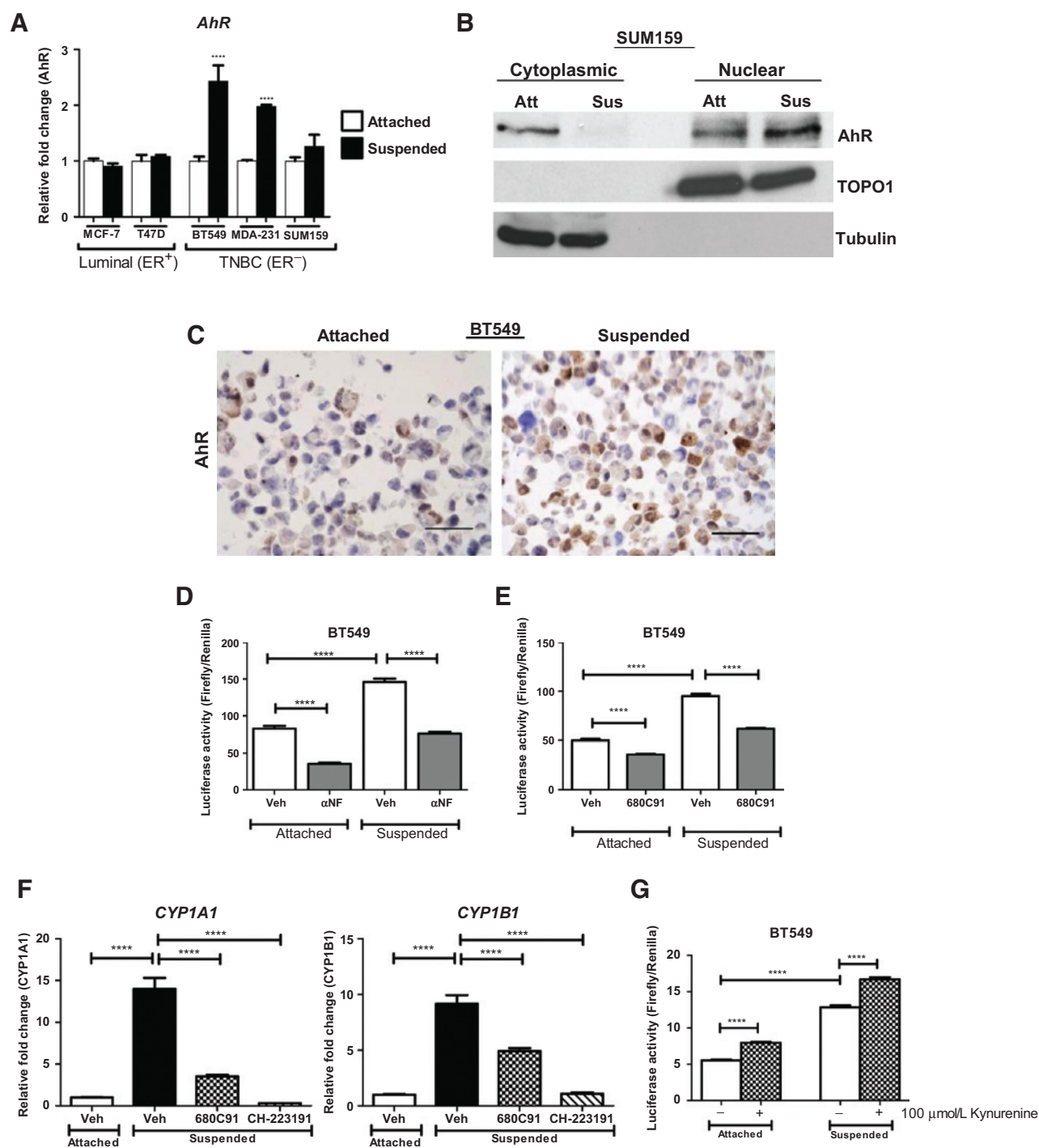
**Figure 1.**

Kynurenine pathway components and activity are increased in TNBC cells in suspension and suppressed by TDO2 inhibition. A, BT549 cells were grown in either attached or forced-suspension conditions on poly-HEMA-coated plates in biologic quadruplicate for 24 hours. RNA was harvested at 24 hours and hybridized onto Affymetrix Human Gene 1.0ST array. Gene expression profile analysis identified genes significantly upregulated in suspension culture ($P < 0.05$). Those involved in tryptophan metabolism and AhR signaling are highlighted in red. B and C, relative TDO2 (B) and KYNU (C) mRNA levels were determined by qRT-PCR in luminal and TNBC cell lines grown in attached or suspended culture for 24 hours. D, Western blot for TDO2, KYNU, and AhR protein in three TNBC cell lines following 24 hours of attached or suspended culture. E, TDO2 protein detected by IHC in BT549 cell pellets grown in attached or suspended culture for 48 hours. Bar, 50 μm. F, secreted kynurenine levels, as measured by HPLC, from BT549 cells grown for 48 hours in the attached versus suspended conditions or suspended plus 10 μmol/L 680C91 (a specific TDO2 inhibitor). *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

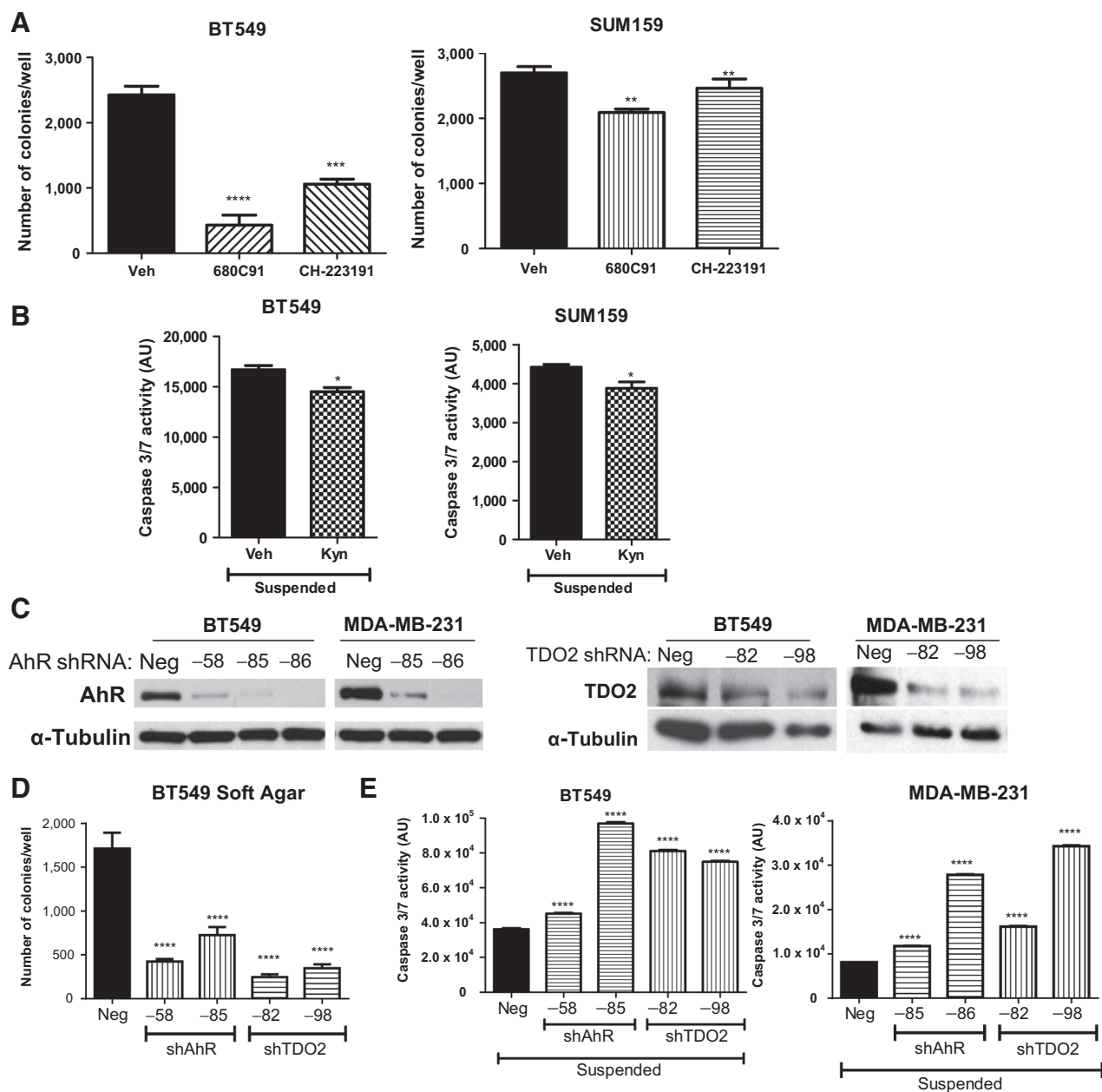
agar (Fig. 3A). To test the effect of Kyn on anoikis resistance, we treated cells in forced suspension culture for 48 hours with Kyn and found that this significantly decreased apoptosis as measured by cleaved caspase activity compared with vehicle control treatment (Fig. 3B). We then performed knockdown of TDO2 and AhR using two shRNA constructs each, and decreased protein expression was

confirmed by Western blot (Fig. 3C). Knockdown of either TDO2 or AhR also significantly decreased growth of BT549 cells in soft agar (Fig. 3D). Knockdown of either TDO2 or AhR also significantly increased apoptosis in BT549 and MDA-MB-231 cells grown in suspension for 48 hours (Fig. 3E), demonstrating that TDO2 and AhR promote survival in anchorage-independent conditions.

D'Amato et al.

**Figure 2.**

AhR expression and localization are affected by suspension culture, and kynurenine activates *AhR* in TNBC cells in suspension. Breast cancer cell lines were plated on uncoated (attached) or poly-HEMA-coated (suspended) tissue culture plates for 24 hours. A, relative *AhR* levels measured by qRT-PCR in ER⁺ or TNBC cells in attached or suspended culture for 24 hours. B, Western blot for *AhR* levels in nuclear and cytoplasmic fractions of SUM-159 cells in attached or suspended culture for 24 hours. C, *AhR* protein as detected by IHC in BT549 cell pellets grown in attached or suspended culture for 48 hours. Bar, 50 μ m. D and E, *AhR* reporter activity in BT549 cells in attached or suspended culture for 24 hours treated with vehicle [0.1% MeOH:EA (3:1); D] or 10 mmol/L α -Naphthoflavone (*AhR* antagonist) or vehicle (0.1% DMSO; E) or 10 μ mol/L 680C91 (TDOi). F, relative expression of *AhR* target genes by qRT-PCR in BT549 cells in attached or suspended culture treated with vehicle, 680C91, or CH-223191 (*AhR* antagonist) for 24 hours. G, *AhR* reporter activity in BT549 cells in attached or suspended culture for 24 hours treated with vehicle (2.5×10^{-4} M HCl) or crystalline kynurenine (100 μ mol/L). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

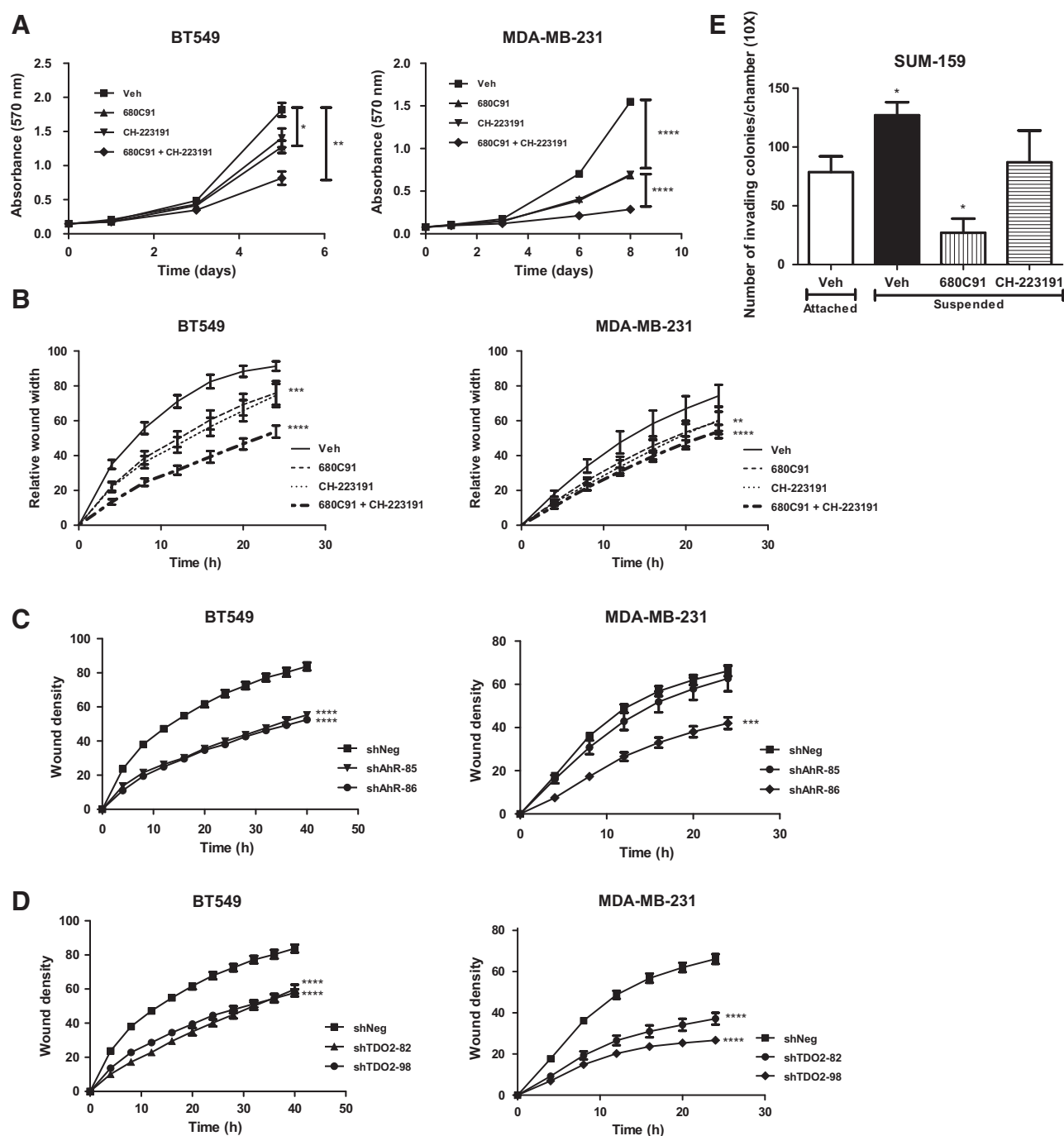
**Figure 3.**

Targeting the kynurenine pathway or AhR increases cell death of TNBC cells in suspension. A, anchorage-independent growth of SUM159 or BT549 cells pretreated with vehicle (0.01% DMSO), 10 μ M/L 680C91 (TDOi), or 10 μ M/L CH-223191 (AhR antagonist) for 24 hours, then plated in 0.25% soft agar where respective treatments were maintained for 18 to 21 days. B, caspase 3/7 activity in TNBC cells (BT549 and SUM159) treated with either vehicle control or 100 μ M/L kynurenine while cultured in suspension for 48 hours. C, Western blot of TDO2 or AhR in attached BT549 or MDA-MB-231 cells following transduction with nontargeting shRNA (shNEG) or shRNA constructs targeting AhR or TDO2. D, anchorage-independent soft-agar growth of BT549 cells with knockdown of TDO2 or AhR grown in soft agar for 14 days. E, caspase 3/7 activity in BT549 or MDA-MB-231 cells transduced with nontargeting shRNA (shNEG) or shRNA constructs targeting AhR or TDO2, and plated in poly-HEMA-coated plates for 48 hours. **, $P < 0.01$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

To determine if TDO2 and AhR also mediate growth of TNBC cells, MDA-MB-231 and BT549 cells were treated with 680C91 or CH-223191, and cell number was measured over time. Both inhibitors significantly decreased the number of cells when grown in traditional attached conditions, and the combination was more effective than either drug alone (Fig. 4A). MDA-MB-231, BT549, and SUM159 cells were also treated

with a range of 680C91 concentrations, and GI50 values were calculated as 20 μ M/L, 61 μ M/L, and 102 μ M/L, respectively (Supplementary Fig. S5). Knockdown of TDO2 or AhR similarly resulted in decreased growth of MDA-231 cells (Supplementary Fig. S6), demonstrating a role for TDO2 and AhR signaling in baseline growth of TNBC cells *in vitro* even in attached culture.

D'Amato et al.

**Figure 4.**

TDO2 and AhR inhibition decreases cell number, migration, and invasion of TNBC cells. BT549 and MDA-MB-231 cells (TNBC) were treated with either vehicle control (0.01% DMSO), 10 $\mu\text{mol/L}$ 680C91 (TDOi), 10 $\mu\text{mol/L}$ CH-223191 (AhR antagonist), or in combination. A, crystal violet assay of BT549 and MDA-MB-231 cells treated for 8 days. B, scratch wound assay of BT549 and MDA-MB-231 cells treated in media containing 0.5% FBS. C and D, scratch wound assay of BT549 and MDA-MB-231 cells transduced with a nontargeting control (shNeg) or shRNAs targeting AhR (shAhR-85, shAhR-86; C) or TDO2 (shTDO2-82, shTDO2-98; D). E, Boyden chamber invasion assay of SUM-159 cells pretreated with either vehicle control (0.01% DMSO), 10 $\mu\text{mol/L}$ 680C91 (TDOi), or 10 $\mu\text{mol/L}$ CH-223191 (AhR antagonist) in the attached or suspended conditions for 24 hours, then seeded into the upper chamber and allowed to invade through a Matrigel-coated membrane. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

In addition, pharmacologic inhibition of TDO2 and AhR each significantly reduced migration of MDA-MB-231 and BT549 cells in a scratch wound assay, and again the combination was more effective than either inhibitor alone (Fig.

4B). Knockdown of either TDO2 or AhR recapitulated this effect, significantly diminishing migration in the scratch wound assay in both BT549 and MDA-231 cells (Fig. 4C and D).

To test whether the increased TDO2 and AhR expression observed in cells grown in suspension affects invasive capacity, SUM159PT cells were grown for 48 hours either in the attached condition, or in forced suspension culture with or without addition of 10 $\mu\text{mol/L}$ of the TDO2 inhibitor 680C91 or the AhR inhibitor CH-223191. After 24 hours, 25,000 viable cells were plated in a Matrigel-coated transwell invasion chamber with continuous treatment. Cells grown in suspension for 24 hours were significantly more invasive than control cells grown in the attached condition (Fig. 4E). The addition of the TDO2 inhibitor 680C91 during suspension culture greatly decreased the invasive capacity of viable cells. Although the cells treated with the AhR inhibitor showed decreased invasion, this effect was not statistically significant (Fig. 4E).

NF- κ B regulates expression of kynurenine pathway genes and AhR in TNBC cells

We previously demonstrated that NF- κ B activity is substantially increased in TNBC cells in suspension, but not in ER⁺ cells (31). Here, we again demonstrate increased NF- κ B activity in TNBC cells in suspension as measured by an NF- κ B luciferase reporter, and this increase is not observed using a reporter containing a mutated binding site (Fig. 5A). Interestingly, a recent global profiling study of ovarian cancer *in vivo* found that knockdown of I κ B-epsilon, a positive regulator of NF- κ B activity, decreased expression of TDO2 and KYNU, suggesting NF- κ B regulation of these genes (36); however, the regulatory mechanism was not directly tested.

To investigate the link between increased NF- κ B activity, TDO2, and KYNU, we treated BT549 cells with a cocktail of TNF α and IL1 β to activate NF- κ B and found that expression of TDO2, KYNU, and AhR (Fig. 5B), as well as the NF- κ B target genes IL6 and IL8 (data not shown), was significantly increased at 24 hours. Next, we tested whether inhibition of NF- κ B in suspension could reduce upregulation of Kyn pathway genes and AhR in TNBC cells grown in suspension. Upstream inhibition of NF- κ B with the I κ B inhibitor PS1145 significantly reduced the suspension-induced increase in TDO2, KYNU, and AhR (Fig. 5C), as well as the NF- κ B target gene IL6 (not shown), demonstrating that NF- κ B activity mediates the increased TDO2, KYNU, and AhR expression in TNBC cells in suspension. Lastly, a constitutively active form of I κ B, a negative regulator of NF- κ B, was expressed in BT549 cells, and this ablated the suspension-induced increase in TDO2, KYNU, and AhR (Fig. 5D). These data demonstrate that the increased transcription of TDO2, KYNU, and AhR in suspension is specifically induced by and dependent on NF- κ B in TNBC.

TDO2 inhibition decreases metastatic colonization *in vivo*

To test the potential contribution of TDO2 activity to the metastatic capacity of TNBC cells *in vivo*, we grew luciferase-expressing MDA-MB-231 cells in forced suspension conditions for 48 hours in the presence of either vehicle control or the TDO2 inhibitor 680C91. A total of 250,000 viable cells, as determined by trypan blue staining, were then injected into the tail vein of NOD/SCID mice, and luminescence was monitored over time. Seven days after injection, mice that received vehicle-treated cells had significantly higher luminescence, and this statistically significant difference was maintained throughout the experiment (Fig. 6A and B). At the conclusion of the experiment at day 28 after injection, lung luminescence was

measured *ex-vivo*, and lungs from mice that received vehicle-treated cells had significantly higher luminescence compared with mice that received cells treated with the TDO2 inhibitor (Fig. 6C). A significant decrease in the number of metastatic nodules in the lungs from mice receiving cells treated with 680C91 was also observed by hematoxylin and eosin (H&E; Fig. 6D). Together, these data demonstrate that TDO2 inhibition decreases the ability of TNBC cells to successfully metastasize following tail vein injection *in vivo*.

TDO2 is more highly expressed in ER⁻ than ER⁺ breast cancer and correlates with poor prognosis

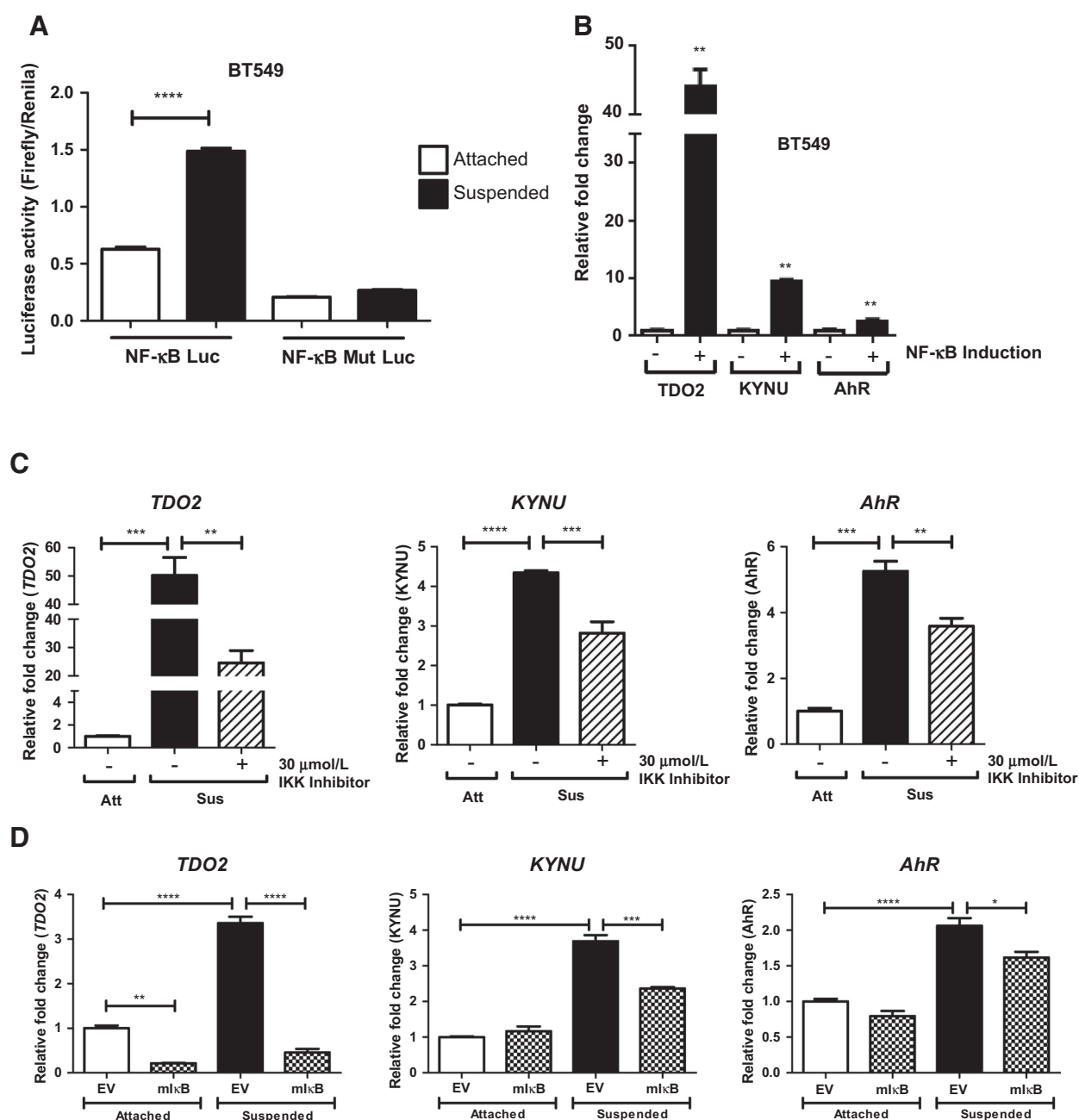
To determine the clinical relevance of our findings, we examined TDO2 expression in primary TNBC samples by IHC. As shown in Fig. 7A, primary TNBC were strongly positive for TDO2 expression. We evaluated TDO2 gene expression in patient samples using publicly available gene expression microarray datasets from Oncomine. The Curtis and colleagues dataset (37) had the largest patient population ($n = 1,998$) and was therefore selected for analysis. Notably, TDO2 was the sixth most highly overexpressed gene in breast carcinoma compared with normal breast tissue (top 1% of overexpressed genes, $P < 0.0001$; Fig. 7B). TDO2 gene expression was significantly higher in ER-negative breast tumors compared with ER-positive tumors ($P < 0.0001$; Fig. 7C). High TDO2 expression was also associated with increasing grade ($P < 0.001$; Fig. 7D), supporting the hypothesis that tumors with high TDO2 expression may have increased metastatic potential. Similarly, IDO1 was overexpressed in breast carcinoma compared with normal breast tissue, although it was not as highly overexpressed as TDO2 (Supplementary Fig. S7A). IDO1 was also more highly expressed in ER⁻ than ER⁺ breast tumors in both the Curtis and TCGA datasets (Supplementary Fig. S7B and S7C).

Finally, patients whose tumors had above-median TDO2 expression had approximately 3 years shorter overall survival when compared with those with below-median TDO2 expression (median overall survival 10.62 years vs. 13.31 years, respectively; $P = 0.0002$, log-rank test; Fig. 7E), suggesting that TDO2 expression may contribute to tumor progression and poor prognosis. Similarly, patients with above-median IDO1 expression had a shorter survival than those with lower IDO1, but the difference in survival was not as large (Supplementary Fig. S7D). This further supports the importance of targeting TDO2 in breast cancer patients, possibly in combination with IDO1, rather than targeting IDO1 alone as in current clinical trials.

Discussion

TNBC has no effective targeted therapies and the worst prognosis of the breast cancer subtypes, due in part to its propensity for rapid metastasis (3). Indeed, patients with metastatic TNBC have a median survival of only 13 months (5). However, few studies have examined pathways that support anoikis resistance in TNBC. In this study, global gene expression profiling revealed the novel finding that TNBC cells in forced suspension upregulate two related pathways: AhR signaling and the kynurenine pathway of tryptophan catabolism. Indeed, global metabolomic profiling identified formylkynurenine and Kyn as the most highly upregulated intracellular metabolites, and Kyn as the third most-highly

D'Amato et al.

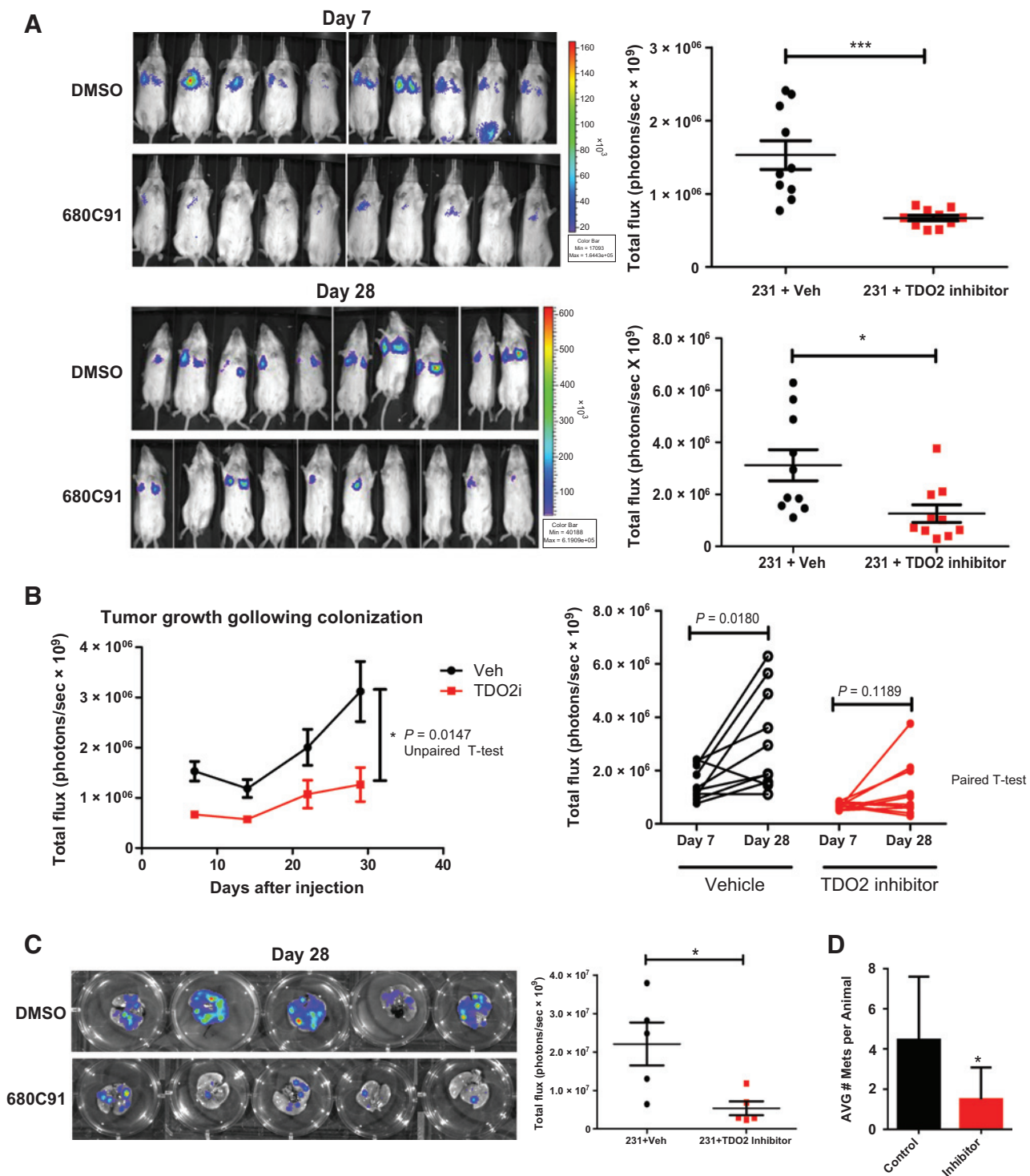
**Figure 5.**

NF-κB regulates expression of TDO2, KYNU, and AhR in TNBC cells in suspension. A, activity of an NF-κB (left) or mutated NF-κB (right) luciferase reporter in BT549 cells in attached or suspended condition for 24 hours. B, relative expression of *TDO2*, *KYNU*, and *AhR* by qRT-PCR in BT549 cells treated with vehicle or TNFα (10 ng/mL) plus IL1β (10 ng/mL) for 24 hours. C, relative expression of *TDO2*, *KYNU*, and *AhR* by qRT-PCR in BT549 cells grown in attached or suspended culture treated with vehicle (0.01% DMSO) or 30 μmol/L PS1145 (IKK inhibitor) for 24 hours. D, relative expression of *TDO2*, *KYNU*, and *AhR* as measured by qRT-PCR in BT549 cells grown in attached or suspended culture and expressing a stable empty-vector (EV) or mutant (constitutively active) IκB expression vector. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by ANOVA with Bonferroni multiple comparison test.

increased secreted metabolite in conditioned media from cells in forced suspension.

Based on our data demonstrating that TNBC cells in suspension upregulate AhR and TDO2, increase secretion of Kyn, and rely on TDO2 and AhR for anchorage-independent growth and invasive potential, we hypothesized that suspended TNBC cells

utilize an autocrine signaling loop in which Kyn activates AhR to support anoikis resistance and anchorage-independent growth, as well as migration and invasion (Fig. 7F). Central to this model is upregulation of TDO2, a key enzyme in the Kyn pathway. Our observation that pharmacologic inhibition of TDO2 decreased the ability of MDA-MB-231 cells to form lung

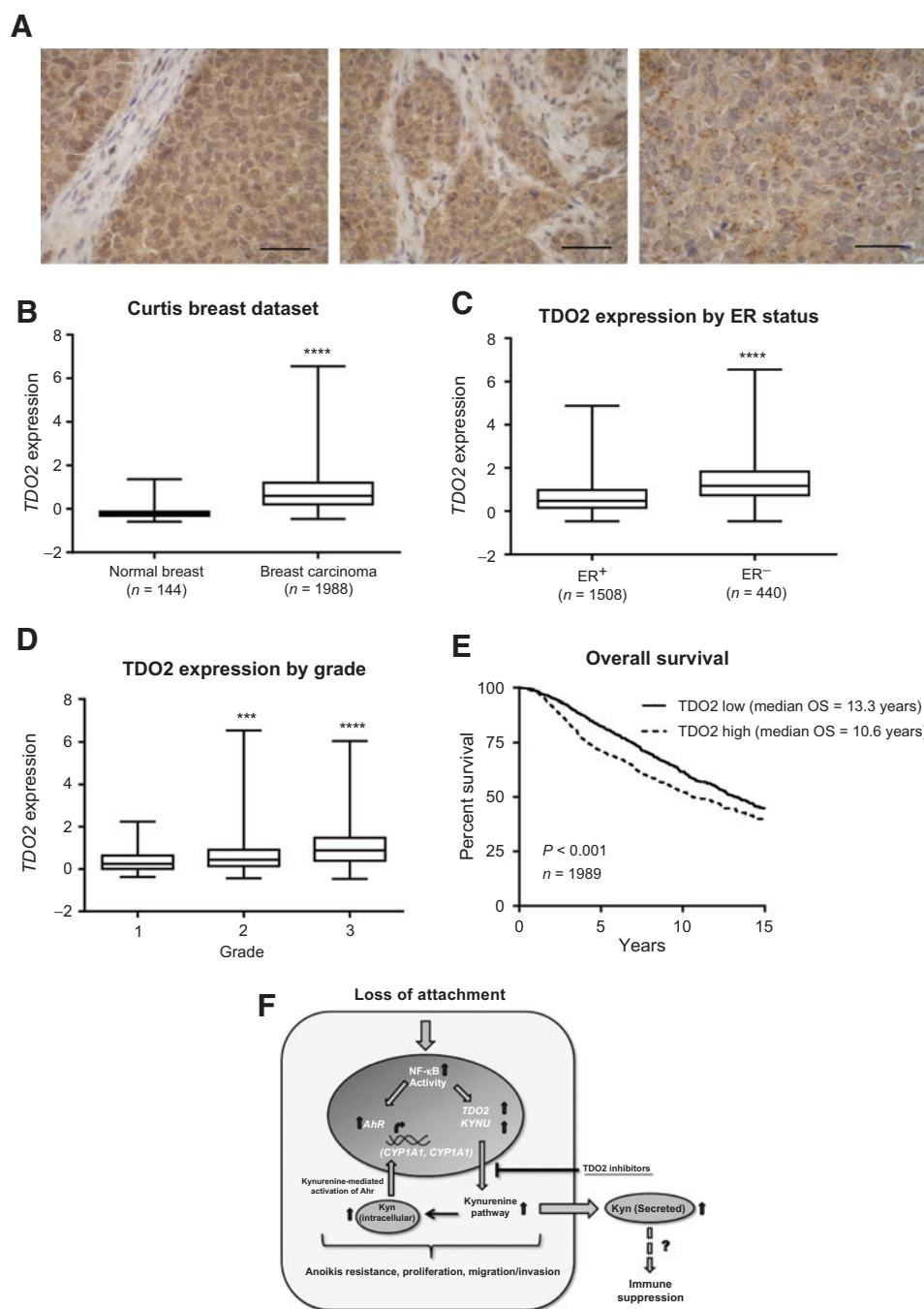
**Figure 6.**

Targeting TDO2 decreases colonization and outgrowth of TNBC cells *in vivo*. Luciferase-expressing MDA-MB-231 cells were pretreated for 48 hours in forced-suspension culture with either vehicle (0.01% DMSO) or 10 $\mu\text{mol/L}$ 680C91 prior to tail vein injection. A and B, metastatic colonization was measured by whole animal IVIS imaging at 7 days after injection and weekly thereafter. C, *ex vivo* imaging of lungs 28 days after injection. D, quantification of lung metastases based on H&E of FFPE lung sections. *, $P < 0.05$; ***, $P < 0.001$ by two-tailed *t* test.

metastases *in vivo* suggests that TDO2 inhibition represents a novel opportunity for targeted therapy to inhibit TNBC metastasis.

Further, we show that the Kyn pathway components TDO2 and KYNU, as well as AhR, are regulated by the transcription factor NF- κB in suspension. We previously identified a different

D'Amato et al.

**Figure 7.**

TDO2 expression in breast cancer clinical samples. A, TDO2 staining in primary TNBC patient samples; bar, 50 μ m. B–E, using the Curtis breast cohort, TDO2 expression was compared between normal breast and invasive ductal carcinoma (B). TDO2 gene expression in breast tumor samples divided by ER⁺ versus ER[−] (C) or by tumor grade (D). E, patients were then split into TDO2-high or -low groups based on median TDO2 gene expression, and overall survival was plotted. F, a model of TDO2-AhR signaling in TNBC cells. NF- κ B activity increases in suspension, increasing expression of TDO2, which leads to increased Kyn (both intracellular and secreted), which subsequently activates AhR and promotes anoikis resistance, migration, and invasion. $P < 0.001$ by log-rank test.

NF- κ B-regulated autocrine signaling loop involving the neurotrophic tyrosine kinase, type 2 (NTRK2 or TrkB), and the TrkB ligand neurotrophin 3 (NTF3) that promotes survival of TNBC cells in suspension (31), suggesting that increased NF- κ B signaling in suspended cells supports multiple prometastatic attributes of TNBC.

In addition to its autocrine role affecting growth, migration, and invasion of tumor cells that we describe here, the Kyn pathway may also facilitate metastasis through paracrine

signaling mechanisms involving suppression of immune surveillance. Specifically, TDO2-mediated Kyn production prevented T-cell-mediated immune rejection of the immunogenic P815 mouse tumor cell line in immunized mice (38). In dendritic cells, IDO1 activity can lead to the AhR-dependent generation of regulatory T cells via a coordinated increase in Kyn and decrease in tryptophan (33). Inhibition of IDO1 also sensitizes cancer cells to chemotherapy in a T-cell-dependent manner (39).

Multiple clinical trials testing the efficacy of Indoximod (1-methyl-D-tryptophan), an IDO1/2 inhibitor, are currently under way, including one in metastatic breast cancer in combination with conventional chemotherapies or immunotherapy (NCT01792050). These trials are based on the ability of IDO1/2 inhibition to reverse immune suppression and enhance the antitumor immune response. However, 1-MT does not target TDO2 (40). Importantly, our data combined with data mined from publicly available breast cancer clinical cohorts suggest that TDO2 may be the more relevant target in TNBC. Interestingly, the membrane protein responsible for tryptophan import into cells, LAT1, is also more highly expressed in TNBC than in other breast cancer subtypes (41). Although a recently published analysis of serum metabolite concentrations in breast cancer patients found no difference in tryptophan levels between ER⁺ and ER⁻ breast cancer patients, median levels of Kyn were significantly higher in patients with ER⁻ tumors (42). Although this finding was attributed to IDO1 activity, we find that TDO2 is higher in ER⁻ tumors than ER⁺ in two publicly available datasets, and that TDO2 expression is higher than IDO1 at baseline in two of three TNBC cell lines tested and TDO2 increases more strongly in suspension in all cell lines. Lastly, and perhaps the strongest indicator of the relative role of TDO2 versus IDO1, blocking TDO2 activity completely prevented the detachment-induced increase in Kyn secretion observed in TNBC cells in suspension (Fig. 1F).

Collectively, our results suggest that in TNBC, TDO2 is an important contributor to Kyn production, and thus should be considered as a therapeutic target. High TDO2 in primary breast tumors from publicly available datasets correlates with shorter overall survival. TDO2 was significantly upregulated in metastases of leiomyosarcoma patients compared with primary tumors (43), and increased KYN expression is associated with metastasis of breast cancer cells to the lung and brain (44, 45). In addition to abrogating the autocrine protumorigenic effects of Kyn, TDO2 inhibition may also inhibit Kyn activation of AhR in immune cells and thereby reduce TNBC immune evasion, but this remains to be tested.

Although we demonstrate a significant increase in Kyn levels in TNBC cells in suspension, it is possible that the effects of TDO2 could be mediated in part through production of other intermediate molecules or NAD⁺ production. However, AhR nuclear localization and transcriptional activity are clearly increased in TNBC in suspension culture, and other recent studies find that AhR affects growth and migration of breast cancer cells (27, 28) and castrate-resistant prostate cancer cells (46). Studies examining AhR expression in breast cancer primary tumors found that higher AhR expression correlates with good prognosis (47). However, our data suggest that AhR expression, nuclear localization, and transcriptional activity may be low until cells begin the metastatic process by detaching from the primary tumor. Thus, AhR in the primary tumor may not reflect its role in supporting the metastatic cascade.

The independent tumor-promoting roles of the Kyn pathway (particularly via IDO1) and AhR signaling have been recog-

nized separately for many years. Recently, the connection of these two pathways via Kyn serving as an AhR ligand was discovered (12, 33–35). Our study demonstrates that the TDO2-AhR signaling axis may be of particular importance in TNBC because it is strongly activated in this highly metastatic breast cancer subtype in response to detachment, and supports anchorage-independent survival and invasive capacity. The combination of protumorigenic autocrine effects and potential immunosuppressive paracrine signaling effects makes this pathway an attractive target for therapeutic intervention. Our findings that inhibition of the Kyn or AhR pathways decreased *in vitro* characteristics associated with multiple steps in the metastatic cascade, and that pharmacologic inhibition of TDO2 decreased TNBC metastasis *in vivo*, suggest that inhibitors of TDO2 may represent an exciting opportunity for targeted therapy for TNBC to decrease mortality from this aggressive breast cancer subtype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: N.C. D'Amato, T.J. Rogers, L.I. Greene, D.R. Cochrane, J.K. Richer

Development of methodology: T.J. Rogers, L.I. Greene, T.G. Nemkov, J.K. Richer

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.C. D'Amato, L.I. Greene, D.R. Cochrane, N.S. Spoelstra, T.G. Nemkov, A. D'Alessandro, J.K. Richer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.C. D'Amato, T.J. Rogers, M.A. Gordon, L.I. Greene, N.S. Spoelstra, T.G. Nemkov, K.C. Hansen, J.K. Richer

Writing, review, and/or revision of the manuscript: N.C. D'Amato, T.J. Rogers, M.A. Gordon, N.S. Spoelstra, K.C. Hansen, J.K. Richer

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.C. D'Amato, N.S. Spoelstra, A. D'Alessandro, K.C. Hansen

Study supervision: J.K. Richer

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A TDO2-AhR Signaling Axis Facilitates Anoikis Resistance and Metastasis in Triple-Negative Breast Cancer

Nicholas C. D'Amato, Thomas J. Rogers, Michael A. Gordon, et al.

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The tryptophan-catabolizing enzyme TDO2 as an immunotherapeutic target in triple-negative breast cancer

Lisa I. Greene¹, Tullia C. Bruno², Jill E. Slansky¹, Virginia F. Borges¹, Jennifer K. Richer¹

¹University of Colorado Anschutz Medical Campus, Aurora CO, ²University of Pittsburgh Cancer Institute, Pittsburgh PA

Background: Cancer immune evasion is a critical feature of aggressive metastatic disease, and infiltration of triple-negative breast cancer (TNBC) tumors by CD8 T cells correlates with improved prognosis. Conversion of tryptophan (trp) into kynurenine (kyn) and the subsequent secretion of kyn may be a mechanism of immune evasion, given that trp depletion triggers T cell death, and that kyn binds the aryl-hydrocarbon receptor (AhR) in immune cells, often with suppressive consequences. Recently, we identified the expression and activity of trp-catabolizing enzyme trp 2,3-dioxygenase (TDO2) as being upregulated in TNBC cells during survival in forced-suspension culture, a model for anchorage independence during the metastatic cascade. TDO2 is normally only expressed in the liver, brain, and placenta. We hypothesize that upregulation of TDO2 in TNBC cells and the subsequent increase in kyn secretion and trp depletion is a critical component of immune evasion in this disease, and that targeting TDO2 could improve anti-TNBC immunity. **Methods:** The TNBC cell line BT549 was plated in attached or suspended conditions for 24 or 28 hours, and conditioned media (CM) was collected. CD8 T cells were isolated from the blood of healthy human donors, activated via CD3/CD28 stimulation, and treated with purified kyn or BT549 CM. **Results:** Purified kyn increased CD8 T cell death at 50 μ M ($p=0.02$) and 100 μ M ($p<0.01$) concentrations and decreased proliferation at 100 μ M ($p=0.02$). Addition of the AhR antagonist CH-223191 (10 μ M) reversed kyn-induced CD8 T cell death ($p=0.02$). Similar to the effect of purified kyn, CM from suspended BT549 cells increased CD8 T cell death ($p<0.01$) and decreased proliferation ($p=0.02$) compared to CM from attached BT549 cells. Furthermore, compared to CD8 T cells cultured in CM from attached BT549 cells, CD8 T cells cultured in CM from suspended BT549 cells produced significantly less γ -interferon ($p<0.01$). Importantly, addition of the TDO2 inhibitor 680C191 (0.1 μ M) to suspended BT549 cells significantly reduced the cell death-inducing effect of this CM on the CD8 T cells ($p=0.03$), suggesting that TDO2 activity is in part responsible for the T cell-suppressive effect of suspended CM. **Conclusions:** Combined with our published data that TDO2 expression and activity increase in anchorage independent TNBC cells, our data suggest that inhibiting TDO2 in metastatic TNBC could improve CD8 T cell-mediated antitumor immunity. **Future Directions:** Our preliminary data indicate that TDO2 becomes aberrantly expressed in TNBC due to loss of the microRNA-200c, and that miR-200c targets multiple additional immunosuppressive pathways (VEGFA, HMOX1, GDF15). These data suggest that loss of miR-200c results in an immunosuppressive reprogramming by permitting tumor cells to hijack mechanisms similar to placental-induced fetal tolerance. Further investigation will determine the importance of these novel findings in TNBC.

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Rogers, Thomas and JK Richer
Department of Pathology, Colorado Anschutz Medical Campus, Aurora CO

Background: Epithelial to mesenchymal transition (EMT) is utilized by carcinoma cells to facilitate metastasis. The microRNA-200 family are potent suppressors of EMT, and loss of miR-200 contributes to the aggressiveness of triple-negative breast cancer (TNBC). Increasing evidence supports a tumor promotional role of the tryptophan catabolism pathway in many solid tumor types, including breast cancer. Tryptophan-2,3-dioxygenase (TDO2) is a rate-limiting enzyme that catabolizes tryptophan into kynurenine (Kyn). Kyn promotes metastasis and immune suppression. We reported that TDO2 is highly expressed in TNBC, and is further upregulated in forced suspension, leading to increased intracellular and secreted Kyn. Kyn activates the aryl hydrocarbon receptor (AhR) in cancer cells via an autocrine loop promoting metastasis by providing a survival signal for tumor cells in transit. We **hypothesize** that TDO2 is regulated both at the transcriptional level by NFkB and the post-transcriptional level by miR-200c and that TDO2 is inappropriately allowed to be expressed as consequence of miR-200c loss in TNBC.

Methods and Results: Restoration of miR-200c to TNBC cells via miRNA mimics or a doxycycline-inducible vector, significantly decreased TDO2 mRNA and protein. Further, miR-200c decreased activity of a luciferase reporter containing the 3'UTR of *TDO2* containing the predicted miR-200c binding site ($P<0.01$). Restoring miR-200c also reduced intracellular and secreted Kyn ($P<0.01$), as measured by UPLC-MS. This decrease in Kyn reduced AhR transcriptional activity ($P<0.01$), as measured by AhR luciferase reporter activity.

Conclusions: We identify a novel mechanism of *TDO2* regulation through targeting by miR-200c. Loss of miR-200c during breast cancer progression may lead to upregulation of TDO2 and subsequent increase in Kyn production, contributing to the ability of TNBC to survive anchorage-independent conditions, suppress the immune system, and rapidly metastasize.

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