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

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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). Cytotoxic T cells require tryptophan and Kyn was recently identified as an endogenous ligand for AhR, a transcription factor that was also upregulated in tumor cells in suspension. 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 catabolism of tryptophan and upregulation of Kyn via the enzyme TDO2 facilitates TNBC survival in transit to metastatic sites and its ability to avoid immune attack by suppressing components of the immune system to mediate the highly metastatic nature of this subtype.					
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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) 100% complete.

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) in the figures mentioned here. 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 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 Rogers T *et al* 2019 paper).

Task 2. Determine if TDO2 or AhR knockdown or inhibition decreases migration (Months 1-4) 100% completed and summarized in first and second year progress report and (D'Amato NC and Rogers T Cancer Research et al 2015 in figures specified, and in the new manuscript Rogers et al published in Jan 2019 (see appendix). 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 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 of D'Amato 2015). TDO2 overexpression vector transduced into luminal (ER+) MCF7 breast cancer cells significantly enhances their ability to grow on soft agar (more anchorage independent) and they produced increased levels of kynurenine (see Figure 2 F and G in our Rogers T *et al* 2019 paper).

Task 3. Determine if TDO2 or AhR knockdown or inhibition decreases invasion (Months 5-8) 100% completed and summarized below 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 Figure 4B D'Amato et al 2015). 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). As mentioned above, a TDO2 overexpression vector transduced into luminal (ER+) MCF7 breast cancer cells significantly enhances their ability to grow on soft agar (more anchorage independent) and they produced increased levels of kynurenine (see Figure 2 F and G in our Rogers T *et al* 2019 paper).

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). 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.

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**) 100% 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 significant difference was maintained throughout (Figure 6A-B).

Task 2. Determine if pharmacological inhibition of TDO2 decreases metastasis following tail vein injection (Months 17-20) 100% Complete and published in Figure 6 of (D'Amato NC et al Cancer Research et al 2015). At the conclusion of the experiment above 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). These data demonstrate that TDO2 inhibition decreases the ability of TNBC cells to successfully colonize the lungs following tail vein injection in vivo.

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 completed. New inhibitors that target both IDO and TDO2 are in the pipeline. We had planned on treating immune competent mice next systemically with 680C91 in their drinking water and then observing if metastasis was affected, but this drug has now been shown to have poor stability *in vivo*. To circumvent this issue, we acquired a second generation TDO2 inhibitor (LM10) f Ludwig Institute for Cancer Research in Belgium through a materials transfer agreement (MTA), but we just acquired a TDO2 inhibitor from Curadev/Roche that is further down the pipeline of development and hope to test that inhibitor in vivo. Meanwhile we are also inhibiting TDO2 with the microRNA-200c, which targets TDO2 as well as other enzymes that produce immune suppressive catabolites as shown in vitro (Rogers T et al Mol Ca Res 2019). **Figures 1 and 2** demonstrate that we can detect TIL in the tumors from this model by flow cytometry (we also monitor peripheral cells and spleen) or examine individual or multiple TIL markers by IHC. We proposed to use two syngeneic mouse (Met-1 and 66Cl-4 cells in two different strains of mice) and measure tumor growth and TILs. We recently generated Met-1 cells with doxycycline inducible miR-200c (**Figure 2**), in which we can monitor effects of targeting of *TDO2* (addition to other EMT associated genes/proteins) on tumor growth and metastasis and begin to examine effects on TIL. We do see an increase in T cells (**Figure 2**) when miR-200c that targets *TDO2* and other genes involved in tumor aggressiveness, and we can now determine if that affects the ability of TNBC models to metastasis and if it is due to miR-200c repressing *TDO2* specifically by adding an expression vector for TDO2 without the miR-200c targeted region that we showed in Rogers et al 2019.

Figure 1. Tumor infiltrating lymphocyte (TIL) and peripheral and spleen lymphocyte detection in mice harboring MMTV-PyMT-derived Met-1 mammary carcinomas by flow cytometry. MMTV-PyMT-derived Met-1 mouse mammary carcinoma cells (2×10^5) were injected into the mammary fat pads (inguinal #4/5) of FVB/NJ mice. At total tumor volume $\sim 500 \text{ mm}^3$ (6 days) tumors (n=5) tumor, blood (pBL) and spleen were analyzed for peripheral lymphocyte analysis.

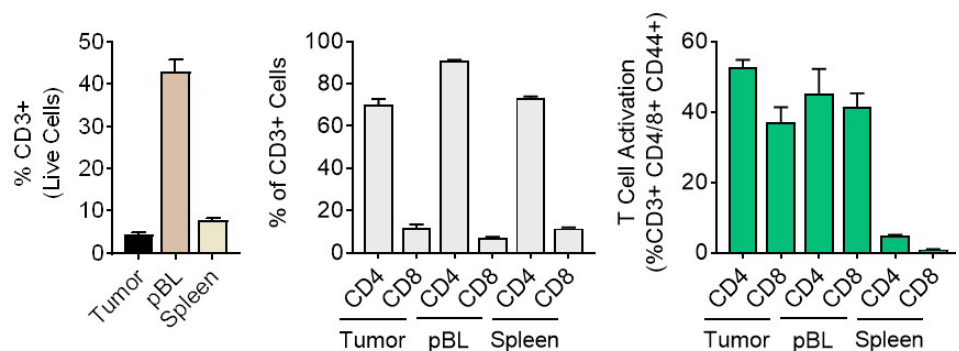
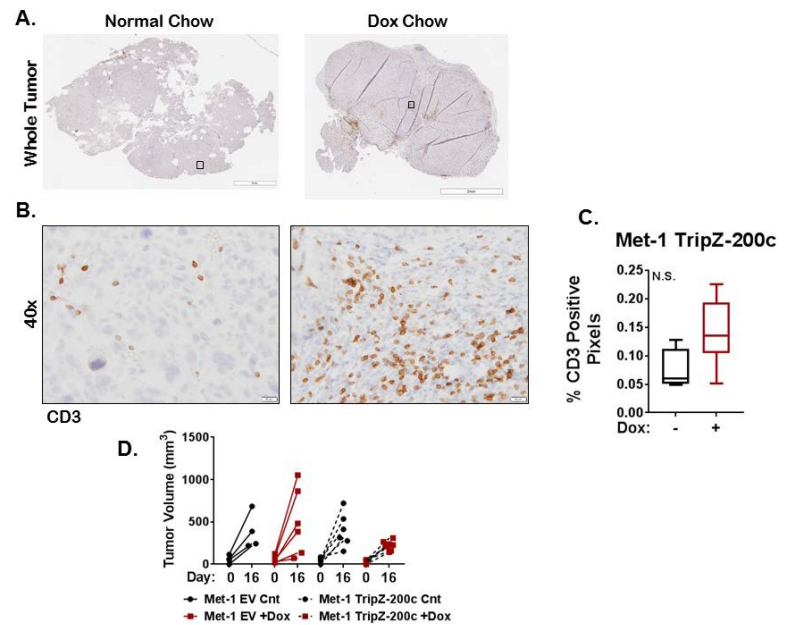


Figure 2. Inducible restoration of miR-200c in the Met-1 model alters tumor growth and may affect TIL. Met-1 TripZ-200c or empty vector control cells were injected into FVB/NJ hosts (1×10^5). Mice randomized and treated $\pm$ Doxycycline when tumors reached $\sim 50 \text{ mm}^3$. Mice were sacrificed and tumors FFPE when the average tumor volume of each group reached 500 mm^3 . **A-B.** CD3 staining and representative images of a whole tumor (A) and at 40x (B). **C.** ImageScope quantification of avg % positive pixels for CD3 in all tumors ($n = 4-8$, unpaired two-tailed T-test). **D.** Rate of tumor growth throughout the experiment.

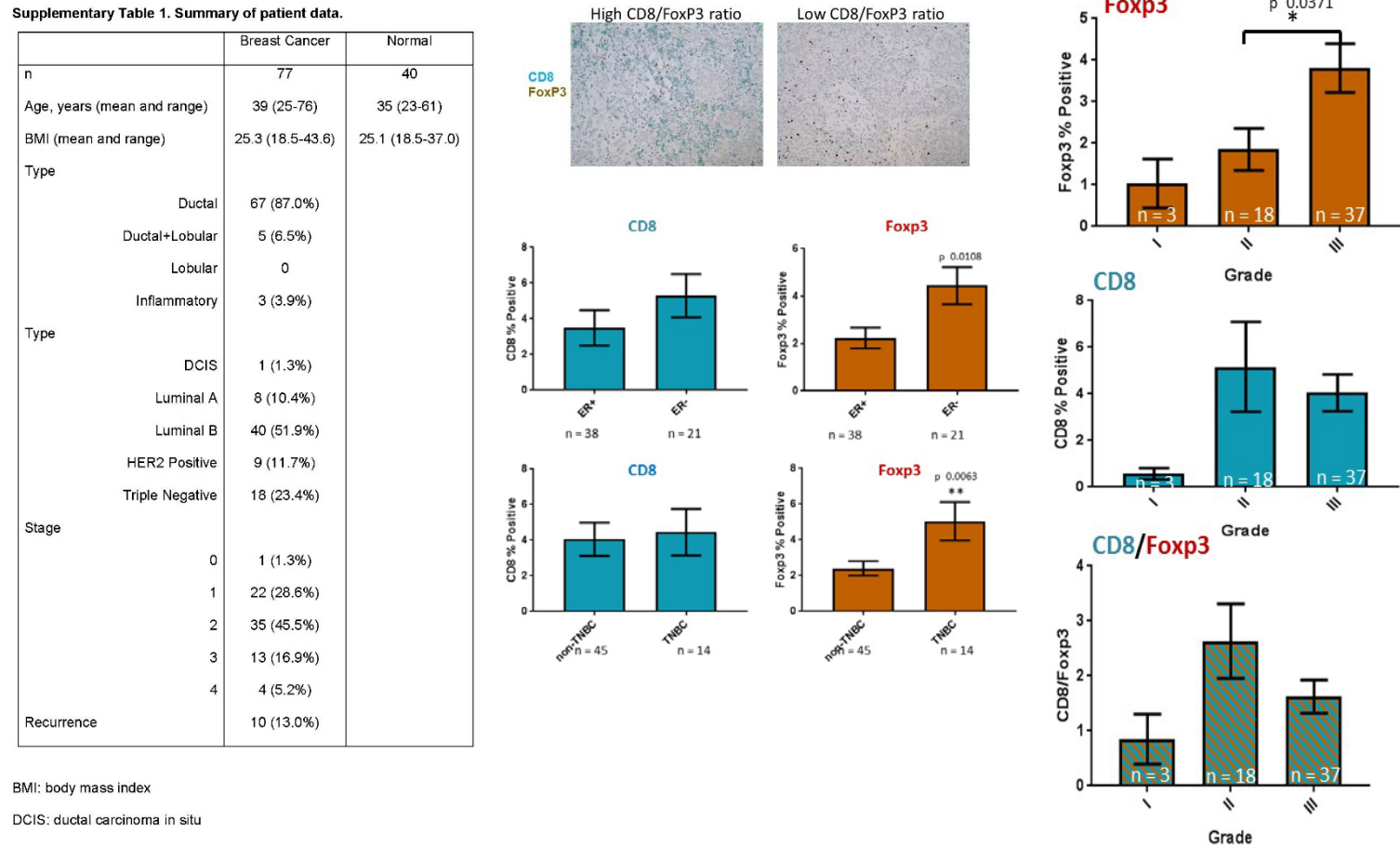


Aim 3: Identify the role of kyn paracrine action in tumor-induced immune modulation via AhR-expressing effector T cells. (Months 1-36) (90% complete) and now in published paper Greene LI et al Mol Ca Research Jan 2019.

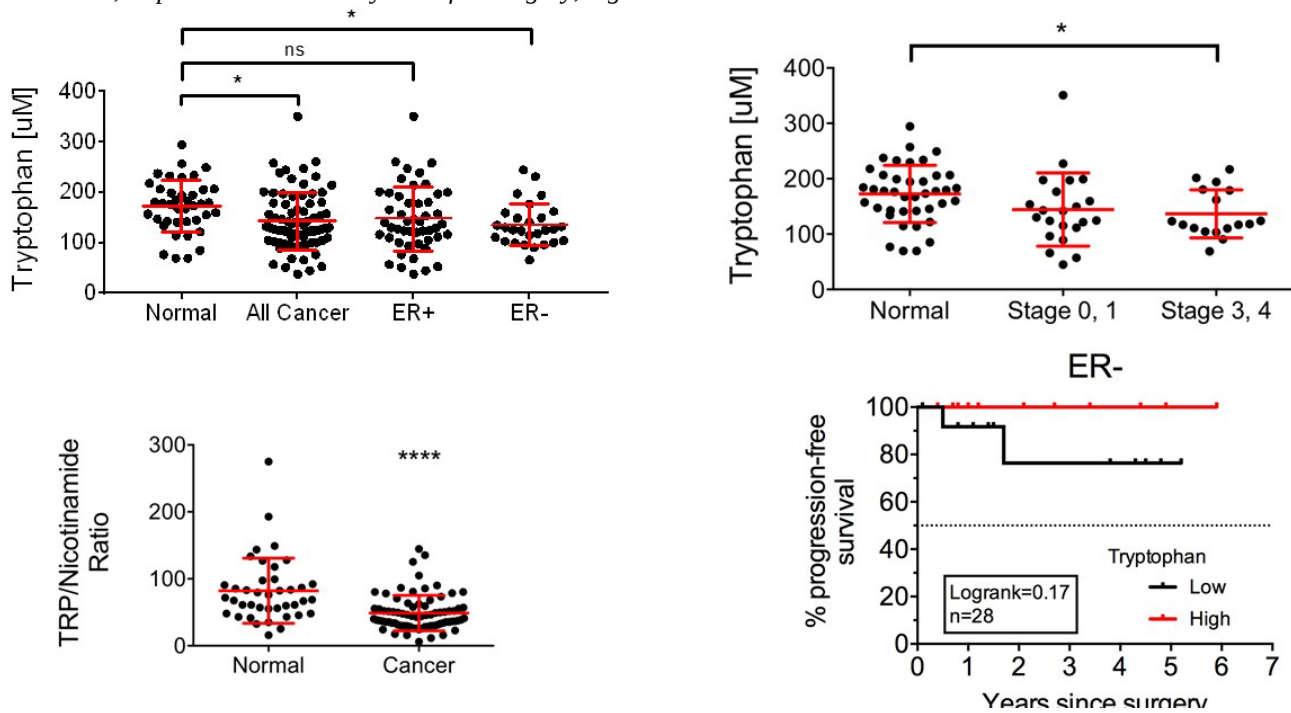
Task 1. Determine whether conditioned media from TNBC in suspension with or without TDO inhibition alters the number and function of effector T cells via the Kyn-AhR pathway (Months 1-15). We determined that TDO2 is upregulated under anchorage independent conditions and by inflammatory signals and as reported above, that it facilitates metastasis. The first paper in the appendix by Rogers et al also shows what allows TDO2 (normally expressed in the liver and placenta) to be expressed in TNBC, but not ER+ breast cancer. The third paper in the appendix from Greene LI et al 2019 in appendix attached shows results for all of the following subtasks below. We first tested the effects of purified kynurenine on primary human T cells isolated from healthy donors and these results are shown in Figure 1 and Figure 2 of the Greene LI *et al* 2019 paper. To test the effect of kynurenine on primary human peripheral blood cytotoxic T cells, CD8 cells isolated from peripheral blood of healthy human donors (Fig. 1A) were treated with increasing doses of kynurenine over a 5-day T-cell receptor (TCR) activation protocol. At 50 and 100 mmol/L kynurenine, CD8 T-cell death increased as measured by a fixable viability dye (Fig. 1B), while CFSE analysis of live CD8 T cells indicated no change in proliferation (Fig. 1C), demonstrating a direct impact of kynurenine specifically on human CD8 T-cell viability. To test whether kynurenine acts through AhR to affect CD8 T-cell viability, CD8 T cells were treated with increasing doses of kynurenine with or without the AhR antagonist CH-223191 (26). Treatment with CH-223191 significantly reduced the cytotoxic effect of 50 mmol/L kynurenine (Fig. 1D and E), indicating that kynurenine acts in part on CD8 T through AhR. We found that conditioned media (particularly from the TNBC cells grown in anchorage independent conditions increases CD8 T-cell viability. Our data also suggest that TDO2 inhibition more so than AhR inhibition contributes to lessening the effect of suspended conditioned media on the CD8 T-cells. 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. BT549 TNBC cells were cultured in attached (ATT) or suspended (SUS) conditions for 24 or 48 hours. Primary human CD8 T cells were cultured in these conditioned media (CM) with CD3 and CD28 antibodies for 5 days. CD8 T cells cultured in SUS CM were less viable than those cultured in ATT CM (Fig. 2A). Because kynurenine decreased IFN γ mRNA in murine cytotoxic T cells (27), we tested whether CM from attached versus suspended TNBC cells would affect primary human CD8 T-cell production of IFN γ . Indeed, the SUS CM significantly reduced IFN γ production compared with ATT CM (Fig. 2B and C). To determine whether the effects of SUS CM were due to increased TDO activity, TNBC cells were plated in ATT or SUS conditions with or without TDO-specific inhibitor 680C91 for 48 hours. While SUS CM again increased CD8 T-cell death, this effect was significantly abrogated when SUS cells were treated with TDO inhibitor 680C91 (Fig. 2D), suggesting that TDO activity is at least partially responsible for the CM effect. However, the CD8 T cells with the AhR antagonist CH-223191 did not rescue the effect of SUS CM, suggesting additional effects of TNBC TDO activity on the CD8 T cells (Fig. 2E) Greene LI *et al* 2019

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). (75% complete). Isolate CD8+ and CD4+ T cells from normal donors and breast cancer patients. Completed. See Figure 1 and 2 in Greene LI et al 2019 in appendix in which we activated T cells by coculture with α CD3 and α CD28 in the presence of conditioned media of TNBC cells grown in either attached condition, suspended, or suspended + 680C91. We 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-cells (Figure 2 in Greene LI et al 2019). We did not yet perform AhR co-staining in CD8+ T cells because in vivo it seems that there is more of a change in FOXP3 (which can suppress the cytotoxic T cells) rather than CD8+ cells in our patient cohort when comparing ER+ versus ER- or TNBC (Figure 3 below). This was completed after graduate student Lisa Greene matriculated so was not in her publication.

Figure 3. Study of immune cell infiltration in breast cancer patients. Blood and FFPE sections of tumor from corresponding patients were acquired from presurgical breast cancer patients under COMIRB and HRPO-approved protocol (COMIRB 15-2225 and HRPO A-18613. Patient characteristics are shown in table at left. Dual IHC was used to stain 62 primary breast tumors for CD8 (Vina Green, appears blue/green in color) and FoxP3 (DAB, appears brown). Middle: example of tumor with high CD8:FoxP3 ratio (indicated by more tumor infiltrating blue cells than brown cells) shown as compared to a tumor with low CD8:FoxP3 ratio 20x magnification. Middle lower panel: FOXP3 was significantly higher in ER negative and TNBC compared to ER+ tumors (t-test). Far right shows that FOXP3 is significantly higher in grade III tumors as compared to lower grade.



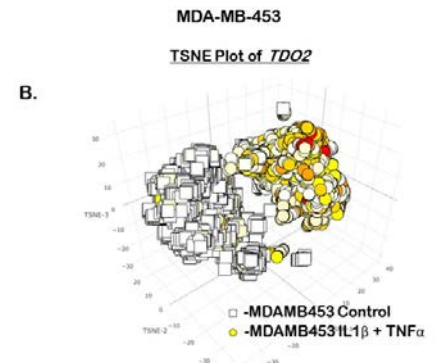
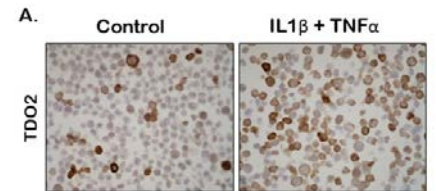
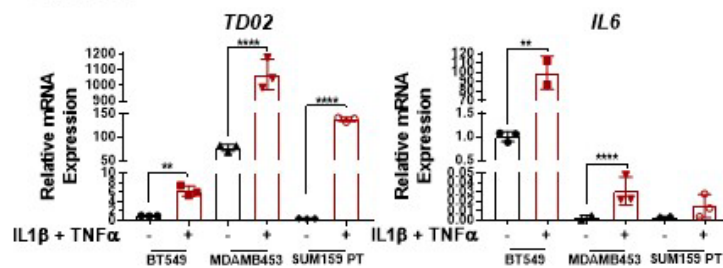
free survival of breast cancer patients with ER negative disease (n=28) where progression was defined by local, regional, or distant recurrence, or patient death in the years after surgery, log-rank test.



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). Received institutional IACUC approval as well as DOD approval. However mouse mammary tumor models Met1 or 66CL4 cells did not appear to make TDO2 in vivo at; however we initiated in vivo experiments so that we can test whether it comes on in late stage tumors or at the metastatic site since it is induced by inflammatory in the human breast cancer cells (Figure 5).

Figure 5. TDO2 mRNA increases in TNBC cell lines following NFkB stimulation. BT549, SUM159 (400,000 cells/well) and MDAMB453 (600,000 cells/well) were plated in 6 well dishes and treated 24 hrs later $\pm$ 10

ng/mL IL-1beta and 10 ng/mL TNFalpha and mRNA harvested 24 hours later. RT-PCR was performed for TDO2 and the inflammatory cytokine IL6 (below). On right - MDA-MB-453 cells treated with either vehicle control or 10 ng/mL IL1beta + 10 ng/mL TNFalpha for 24 hours and immunohistochemistry for TDO2 and harvested for single cell RNA sequencing in which over 3,000 cells were captured/sample and sequenced at a depth of $\sim$ 70,000 reads/cell and TSNE plot illustrates TDO2 expression after stimulation (square = control treated cells; circle = IL1 β + TNF α treated cells).



Task 4. Determine whether tryptophan or kynurenine effects the cytotoxic function of CD8+ T-cells in vivo by inhibiting TDO2 (Months 27-30) We waited on the in vivo experiment to identify the best TDO2 inhibitor to use in vivo. The drugs in clinical trials currently target IDO and not TDO2. However, as mentioned above, new dual inhibitors are coming down the pipeline into clinical trials (see table in review published by Lemos H in Nature Communications 2019). We just obtained one from Curedev/Roche that targets both IDO and TDO2.

Opportunities for training and professional development the project provided:

Two graduate students, Thomas Rogers and Lisa Greene are the graduate students that worked on this project. They both obtained NIH NCI F31 Fellowships (see write-up below). After they left a postdoctoral fellow, Michelle Williams, who obtained her doctorate from Vanderbilt in 2017 started in my lab and took up this project and helped finish experiments to get the Rogers 2019 paper published. Dr. Williams just found out that she scored in the 10th percentile on her NIH NCI F32 fellowship.

Thomas Rogers, Cancer Biology Doctoral Candidate 5th yr, had 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 finished his doctorate degree in my lab. Thomas Rogers interviewed the following prestigious postdoctoral positions: Dr. Costas Lyssiotis- University of Michigan, Dr. Jason Locasale- Duke University, Dr. Dan Nomura- University of California-Berkeley and Dr. Ralph DeBerardinis- UT Southwestern. He chose the latter, who is very well regarded in the field of tumor metabolism. Thomas defended his dissertation at the end of June 2017 and moved to Dallas to start his postdoctoral fellowship.

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. She defended her dissertation on this topic in 2018 and decided to go to medical school. After being accepted at 5 different schools she decided to attend Northwestern University Feinberg School of Medicine in the fall of 2018 and is now finished with her first year.

How were the results disseminated to communities of interest?

Jan 2018	UT Southwestern, Dallas, TX- Green Center for Reproductive Biology “TNBC Hijack Mechanisms of Immune Tolerance”
May 2018	Gordon Research Conference- Mammary Gland Biology and Cancer , Lucca, Italy "Breast Cancer Hijacks a Program of Immune Suppression: Hormonal and Inflammatory Mediators"
Oct 2018	Univ of Colorado Molecular Biology Graduate Program Retreat Talk “miRNA and Immune Evasion”
Feb 2019	MD Anderson - Symposium on Factors Impacting Immune Microenvironment – “Carcinomas Hijack a Trophoblast-Like Program of Immune Suppression”

What do you plan to do during the next reporting period to accomplish the goals? This is the final report for this grant. We are now applying for an invited Expansion Award due Sept 5, 2019

4. IMPACT:

- o **What was the impact on the development of the principal discipline(s) of the project?** Our research strongly suggests 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.
- o **What was the impact on other disciplines?** It was not well known that microRNA can affect tumor metabolism and tumor derived immune suppressive factors.
- o **What was the impact on technology transfer?**

- This project is likely to have an effect 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, but not TDO2. These enzymes are non-homologous - they evolved separately to do the same function (catabolize tryptophan). The first clinical trials targeting IDO did not show efficacy in improving response to immune checkpoint inhibitors (these were in multiple late stage solid tumors), but our data suggests that targeting TDO2 may be more important, at least in triple negative breast cancer.

What was the impact on society beyond science and technology? Gave 3 trainees great opportunities to present their work and further their career development through going on to obtain NIH Fellowships. Now one is doing a postdoctoral fellowship at UT Southwestern in Dallas, TX and one is in medical school Feinberg School of Medicine, Northwestern University Chicago IL

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change. Pharmacologic inhibitors for TDO2 were behind those for IDO in commercial development and are just now becoming available.

- o **Actual or anticipated problems or delays and actions** *None*
- o **Changes that had a significant impact on expenditures** *None*
- o **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.** *None*
- o **Significant changes in use or care of human subjects.** *None*
- o **Significant changes in use or care of vertebrate animals.** *None*
- o **Significant changes in use of biohazards and/or select agents.** *None*

6. PRODUCTS:

- o **Publications, conference papers, and presentations.** The manuscript listed below was a result of this project and two submitted manuscripts are attached in the appendix and the presentations are listed above

Journal publications:

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.

Rogers TJ, Christenson JL, Greene LI, O'Neill KI, Williams MM, Gordon MA, Nemkov T, D'Alessandro A, Degala GD, Shin J, Tan AC, Cittelly DM, Lambert JR, **Richer JK.** Reversal of Triple-Negative Breast Cancer EMT by miR-200c Decreases Tryptophan Catabolism and a Program of Immunosuppression. *Mol Cancer Res* 2019. Jan 17(1): 30-41. PMID: 30213797

Greene LI, Bruno TC, Christenson JL, D'Alessandro A, Culp-Hill R, Torkko K, Borges VF, Slansky JE, **Richer JK.** A Role for Tryptophan-2,3-dioxygenase in CD8 T-cell Suppression and Evidence of Tryptophan Catabolism in Breast Cancer Patient Plasma. *Mol Cancer Res* 2019 Jan 17(1):131-139. PMID:30143553

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

- o **What individuals have worked on the project?** During the last year (which was a no cost extension the following people were supported:

4/2018-3/2019	% effort	Calendar months		
Richer	3%	0.3970146	May-July	10%
SPOELSTRA	0.045603	0.5472306	April	50%
Williams, Michelle	0.134569	1.6148244	May-June	100%

- o **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
- o **What other organizations were involved as partners?**
 - No

8. SPECIAL REPORTING REQUIREMENTS N/A

- 9. **APPENDIX:** Two manuscripts accepted since the last annual report (Rogers et al Mol Can Res) and Greene LI et al 2019) are attached in the appendix as is the first one from 2015 since it is referenced in this report as well.

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

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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.

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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.

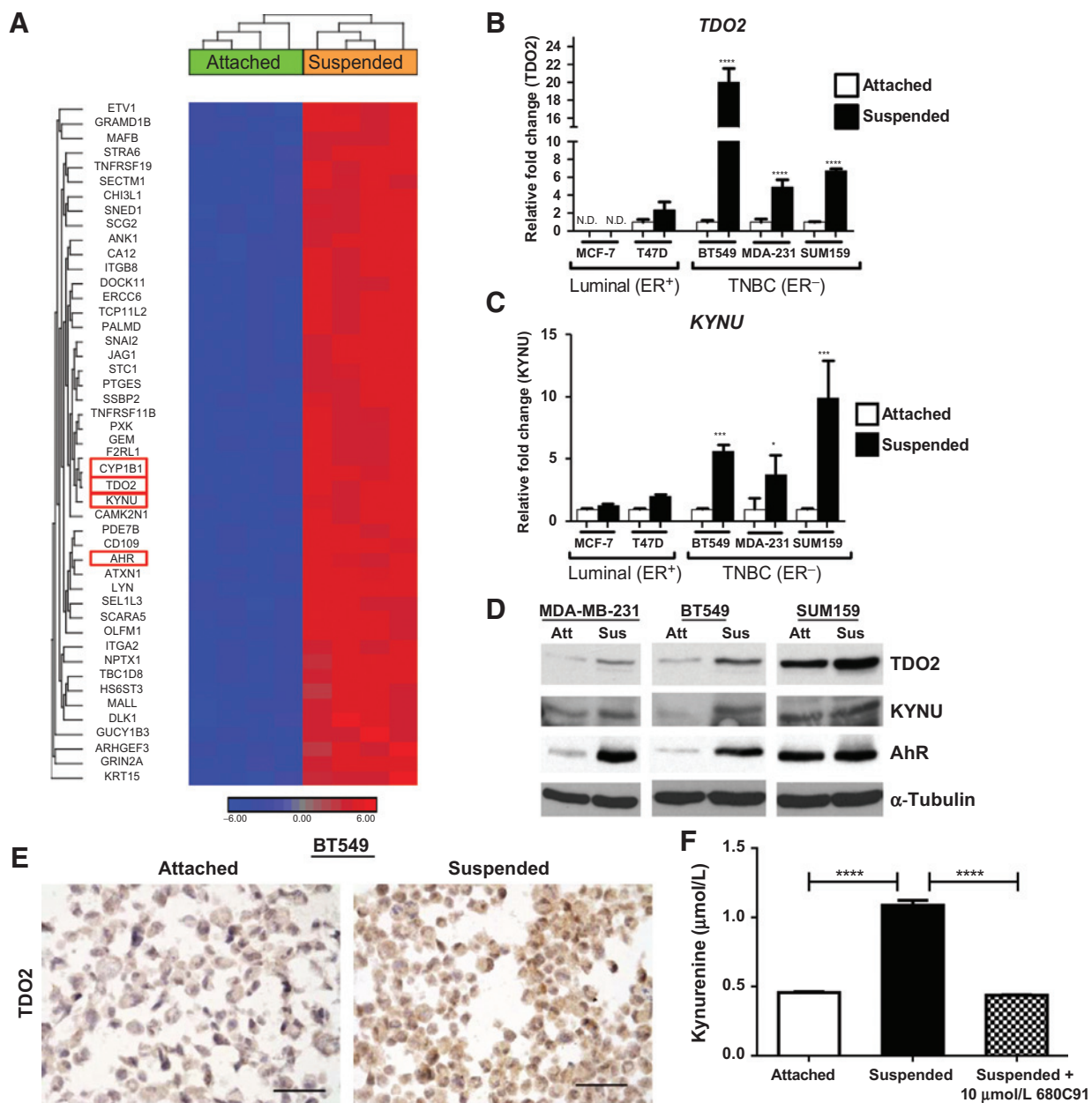
We assessed *AhR* transcriptional activity using a luciferase reporter containing six dioxin response elements (DRE), the consensus *AhR*-binding site. In BT549 and MDA-231 cells, luciferase activity was upregulated more than 2-fold in suspended compared with attached cells (Fig. 2D). The *AhR* antagonist 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

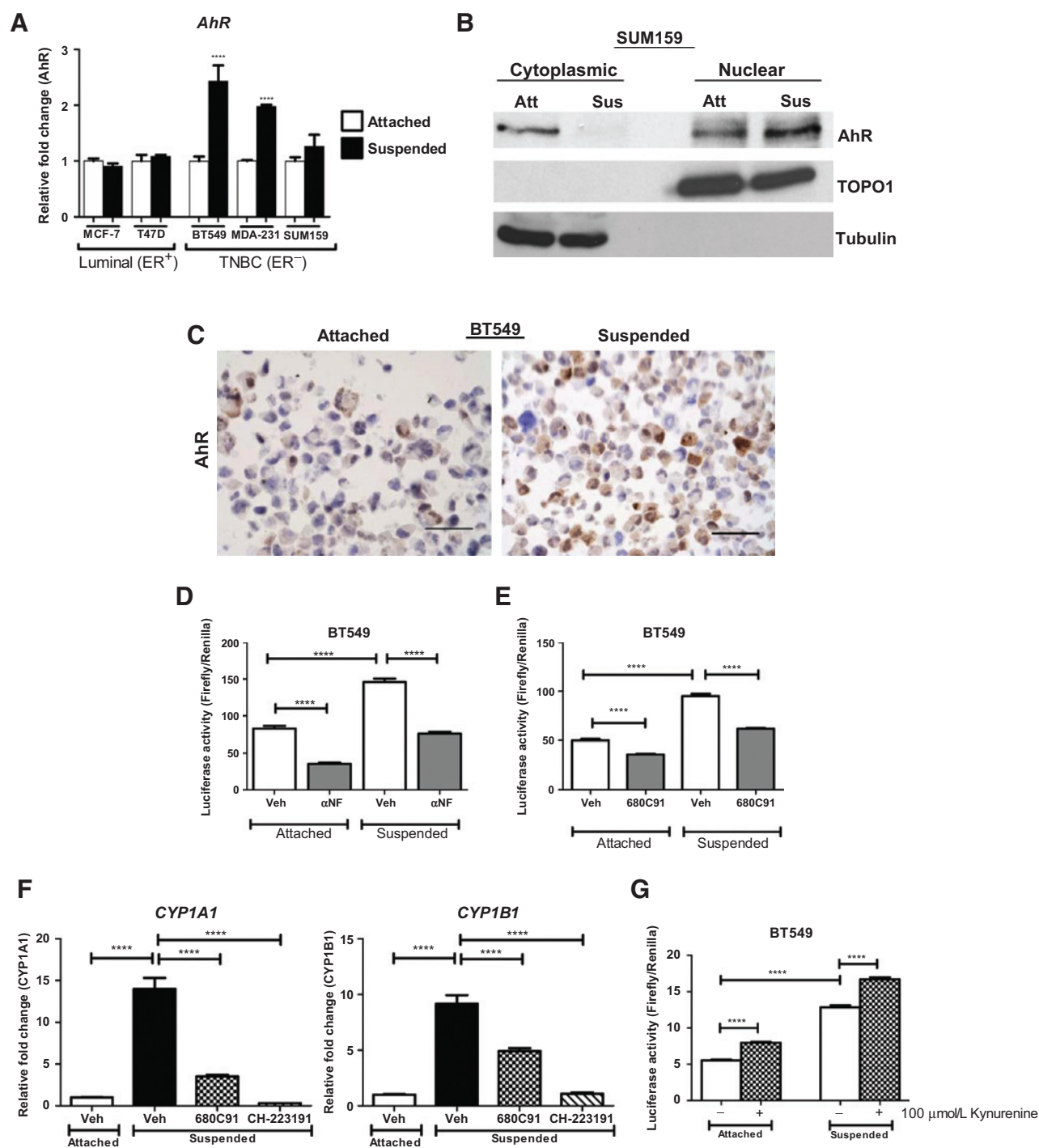
**Figure 1.**

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.

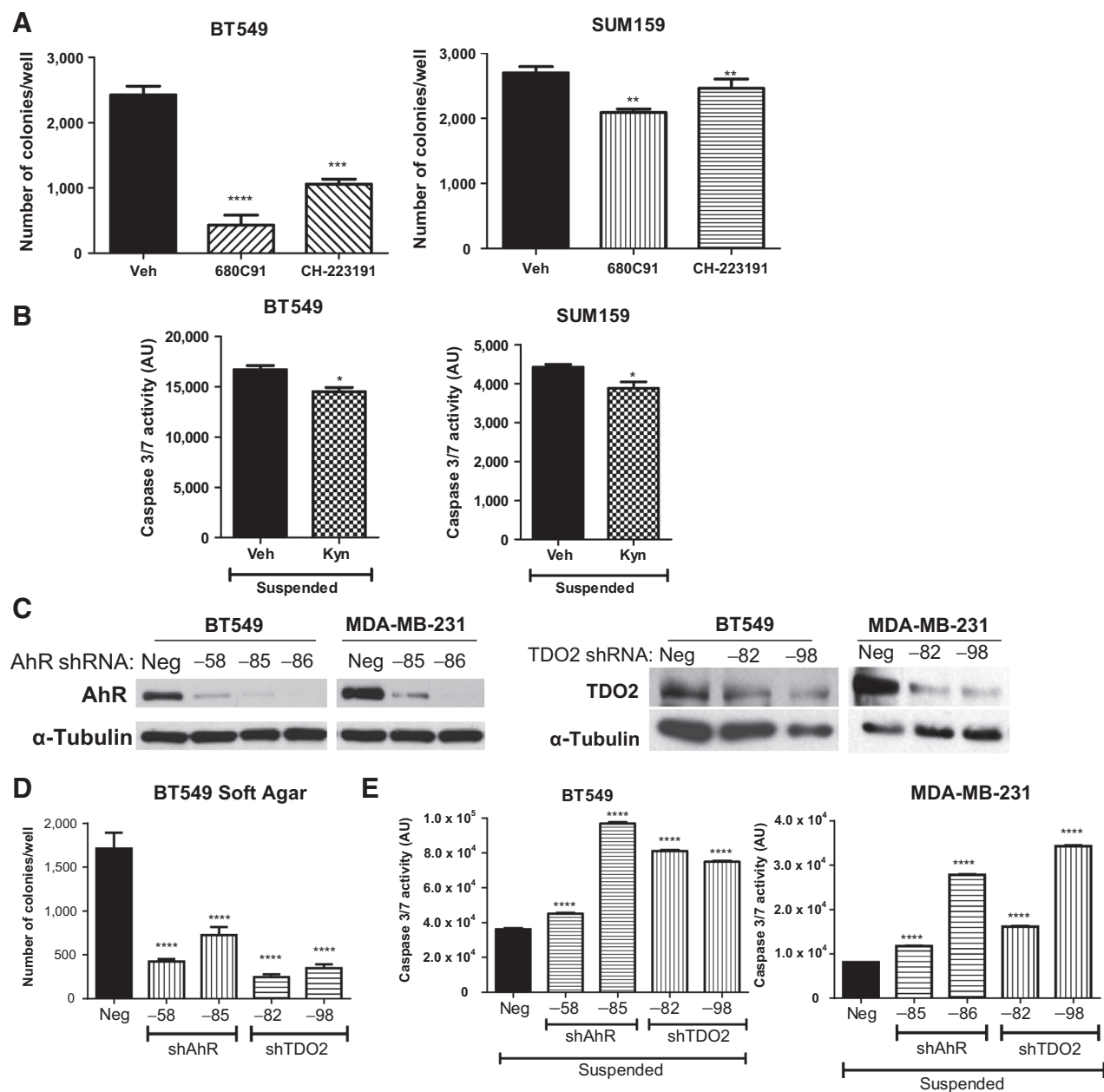
agar (Fig. 3A). To test the effect of Kyn on anoikis resistance, we treated cells in forced suspension culture for 48 hours with Kyn and found that this significantly decreased apoptosis as measured by cleaved caspase activity compared with vehicle control treatment (Fig. 3B). We then performed knockdown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

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**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.

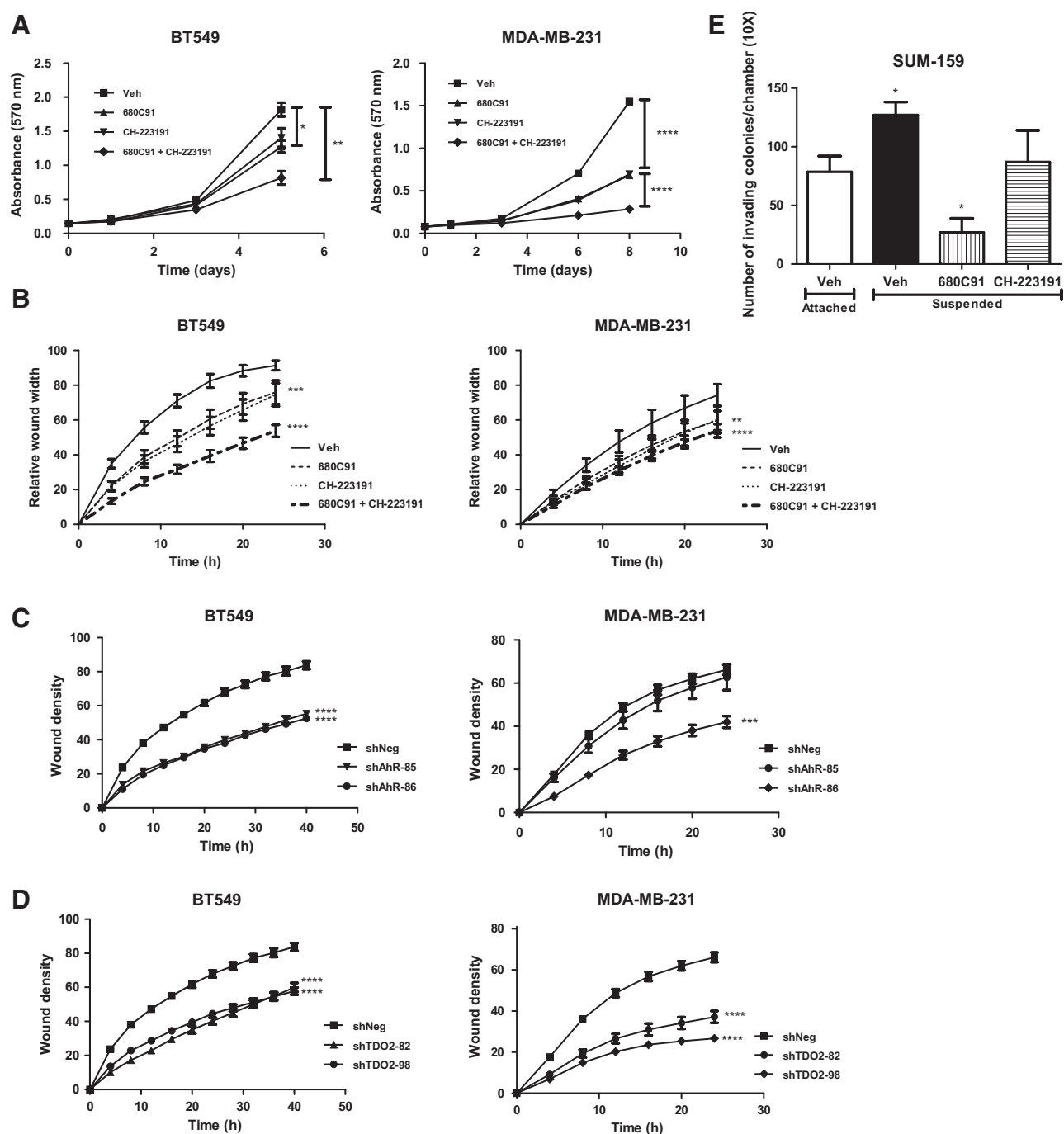
**Figure 3.**

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.

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**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 μ mol/L 680C91 (TDOi), 10 μ 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 μ mol/L 680C91 (TDOi), or 10 μ 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

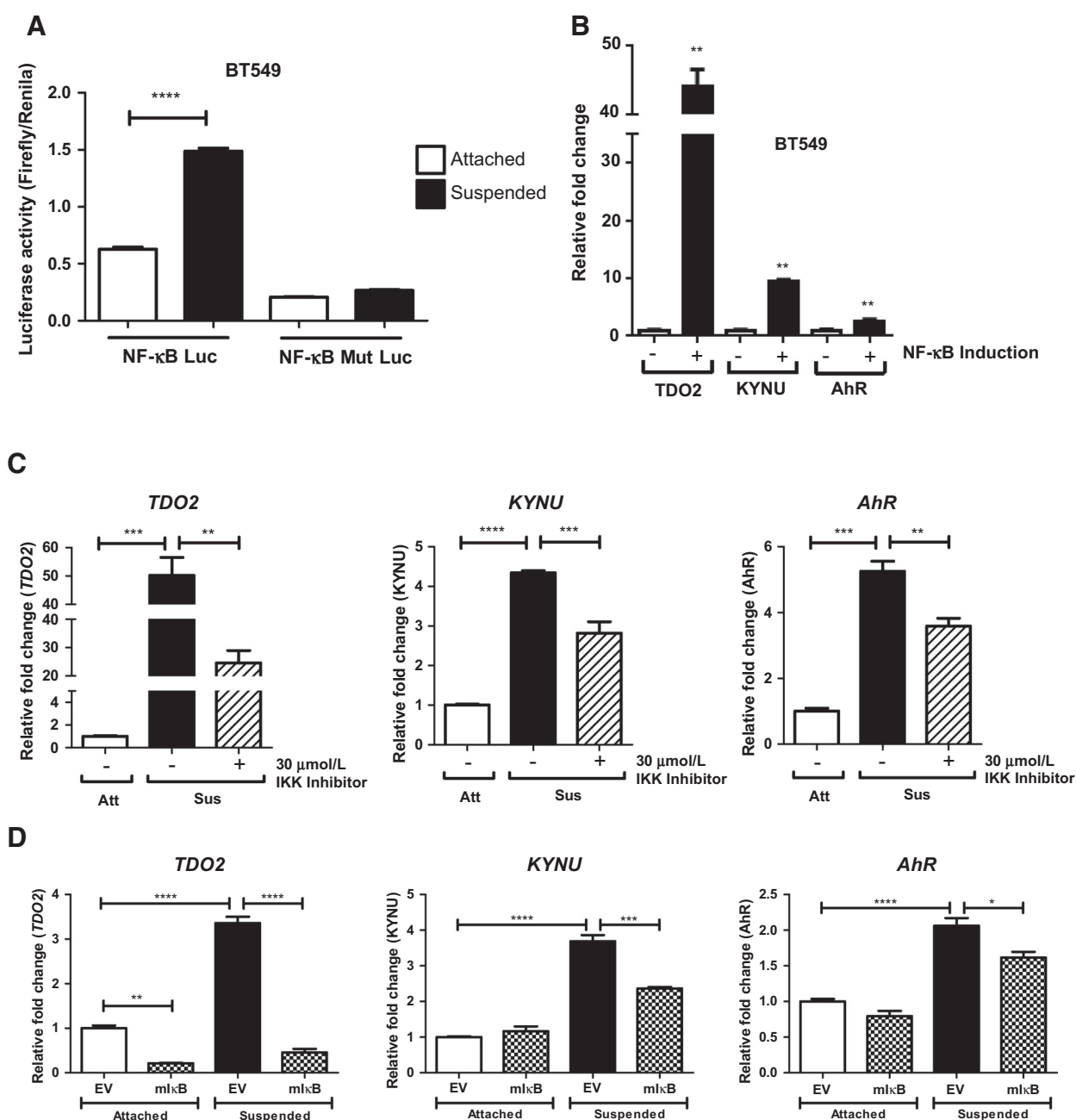
To determine the clinical relevance of our findings, we examined TDO2 expression in primary TNBC samples by IHC. As shown in Fig. 7A, primary TNBC were strongly positive for TDO2 expression. We evaluated TDO2 gene expression in patient samples using publicly available gene expression microarray datasets 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

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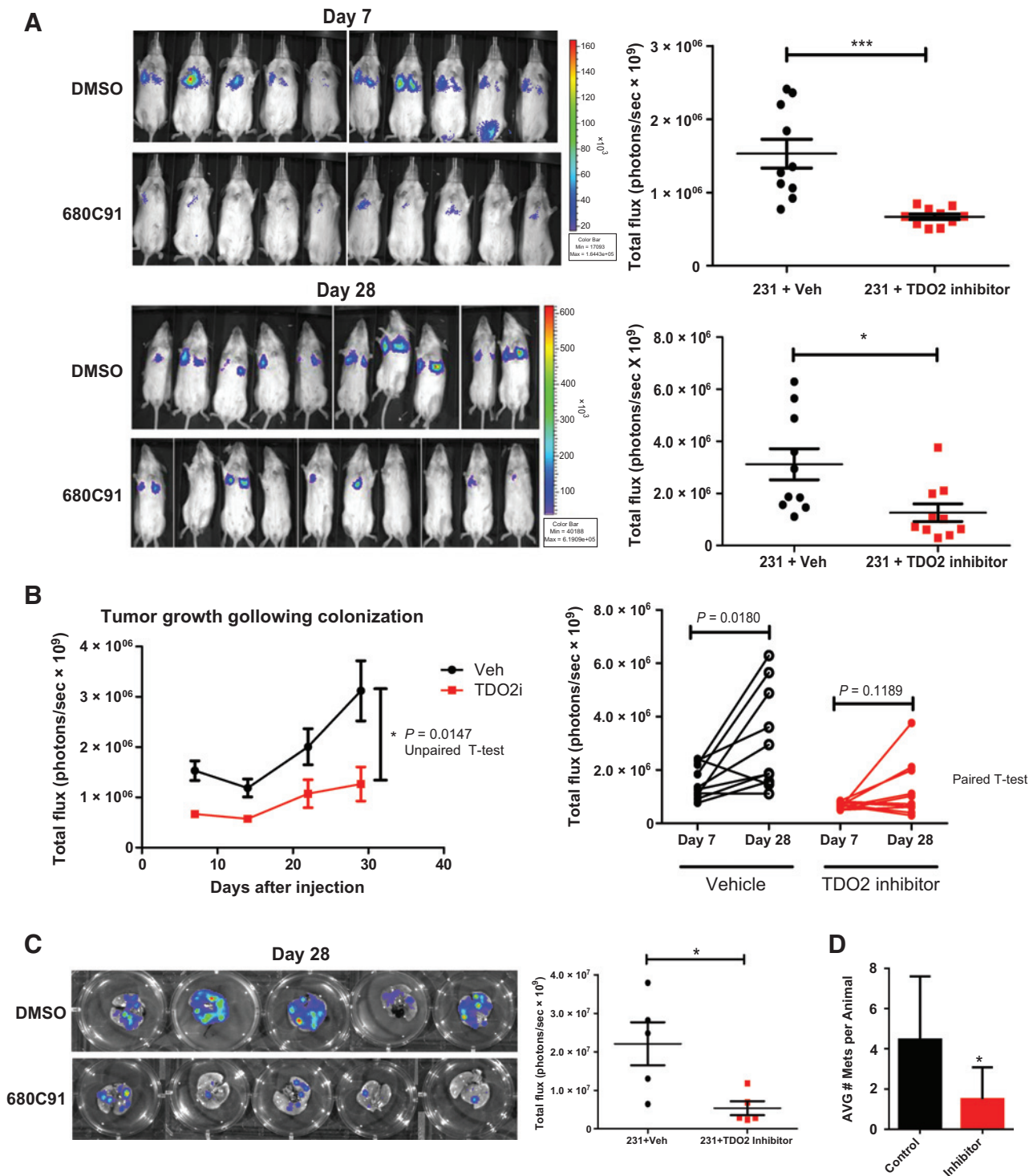
**Figure 5.**

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

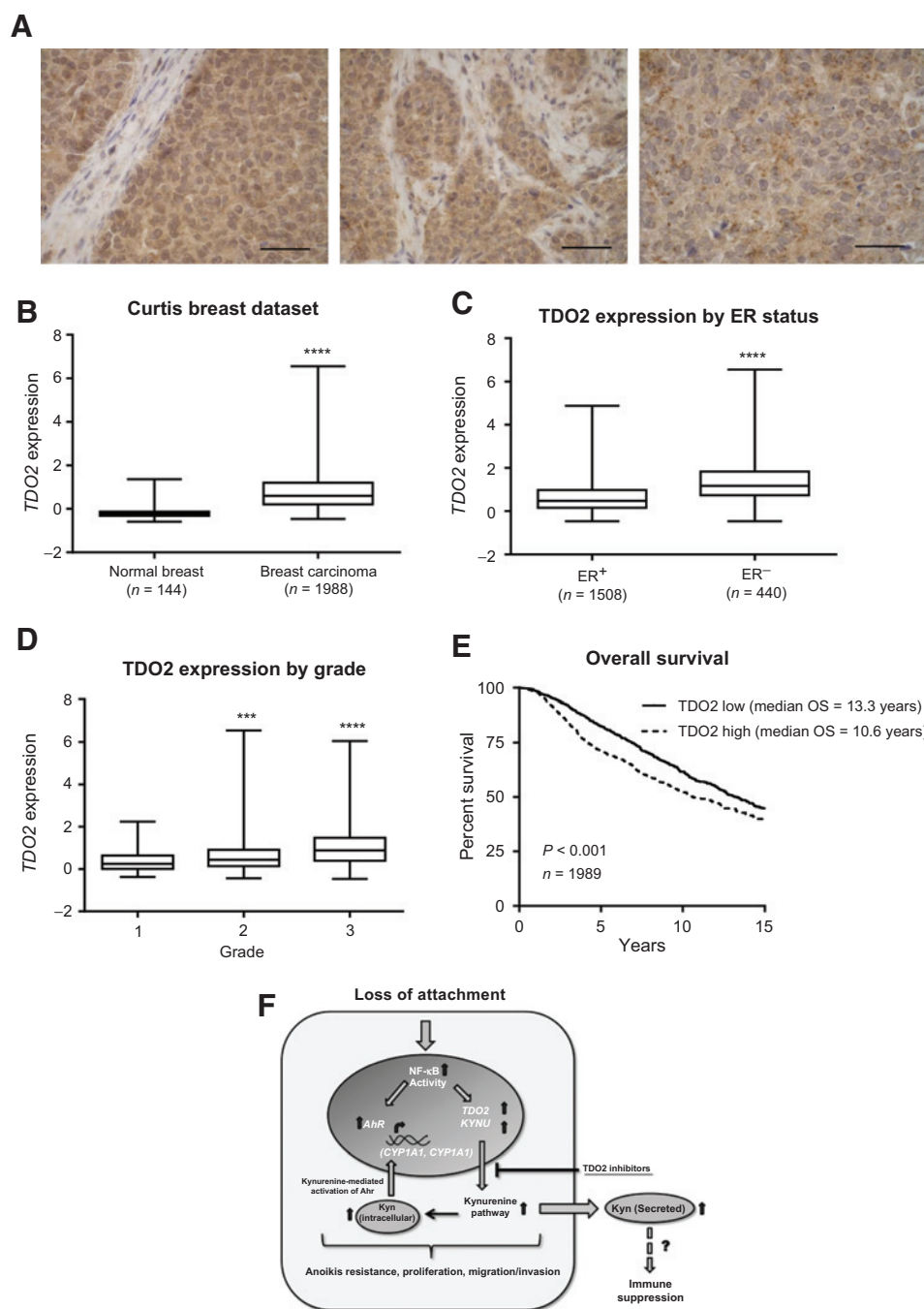
**Figure 6.**

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

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**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

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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

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A Role for Tryptophan-2,3-dioxygenase in CD8 T-cell Suppression and Evidence of Tryptophan Catabolism in Breast Cancer Patient Plasma

Lisa I. Greene¹, Tullia C. Bruno², Jessica L. Christenson¹, Angelo D'Alessandro³, Rachel Culp-Hill³, Kathleen Torkko¹, Virginia F. Borges⁴, Jill E. Slansky⁵, and Jennifer K. Richer¹



Abstract

Tryptophan catabolism is an attractive target for reducing tumor progression and improving antitumor immunity in multiple cancers. Tumor infiltration by CD8 T cells correlates with improved prognosis in triple-negative breast cancer (TNBC) and a significant effort is underway to improve CD8 T-cell antitumor activity. In this study, primary human immune cells were isolated from the peripheral blood of patients and used to demonstrate that the tryptophan catabolite kynurenine induces CD8 T-cell death. Furthermore, it is demonstrated that anchorage-independent TNBC utilizes the tryptophan-catabolizing enzyme tryptophan 2,3-dioxygenase (TDO) to inhibit CD8 T-cell viability. Publicly available data revealed that high *TDO2*, the gene encoding TDO, correlates with poor breast cancer clinical outcomes, including overall survival and distant metastasis-free survival, while expression of the gene encod-

ing the more commonly studied tryptophan-catabolizing enzyme, *IDO1* did not. Metabolomic analysis, using quantitative mass spectrometry, of tryptophan and its catabolites, including kynurenine, in the plasma from presurgical breast cancer patients ($n = 77$) and 40 cancer-free donors ($n = 40$) indicated a strong correlation between substrate and catabolite in both groups. Interestingly, both tryptophan and kynurenine were lower in the plasma from patients with breast cancer compared with controls, particularly in women with estrogen receptor (ER)-negative and stage III and IV breast cancer.

Implications: This study underscores the importance of tryptophan catabolism, particularly in aggressive disease, and suggests that future pharmacologic efforts should focus on developing drugs that target both TDO and IDO1.

Introduction

Triple-negative breast cancer (TNBC) is an aggressive disease that lacks FDA-approved targeted therapies for the majority of patients (1). Importantly, TNBCs with high levels of immune infiltrate have more favorable prognoses (2, 3). Specifically, a high ratio of tumor-infiltrating cytotoxic CD8 T cells to immunosuppressive FoxP3⁺ T regulatory cells correlates with improved breast cancer-specific survival in chemoresistant TNBC (4). Indeed, TNBC has high expression of programmed cell death 1-ligand 1 (PD-L1) compared with other breast cancer subtypes, suggestive of evasion from immune attack (5–7). Active clinical trials are

investigating the efficacy of targeting the PD-1/PD-L1 axis and other immune-directed interventions as treatments for TNBC; however, results in patients with TNBC treated with the anti-PD-1 agent pembrolizumab indicate promising but limited efficacy (8, 9).

We previously discovered that aberrant expression and activity of the hepatic tryptophan-catabolizing enzyme tryptophan-2,3-dioxygenase (TDO) in TNBC cell lines supports anchorage-independent cell growth and late-stage metastasis through an autocrine mechanism, whereby the tryptophan catabolite kynurenine activates the aryl-hydrocarbon receptor (AhR) in the breast cancer cells (10). However, it is clear that kynurenine also has suppressive paracrine effects on immune cells (11, 12). Most studies have focused on the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO) in cancer, and current inhibitors of this pathway in clinical trials only target IDO activity. *IDO* transcript correlates with poor prognosis and increased microvessel density in breast cancer (13). However, *TDO2* (the gene encoding TDO), is significantly higher in estrogen receptor (ER) negative (–) compared with ER⁺ breast tumors and correlates with overall survival in patients with breast cancer, suggesting a potential failure of current tryptophan-catabolizing agents in effectively targeting tumor cell enzymatic activity (10). Importantly, recent negative clinical trial results in melanoma (the ECHO-301 trial) and pancreatic cancer (ECHO-203) suggest that IDO inhibitors are not sufficient for the reversal of tumor immune evasion (14) and indicate that it may be necessary to target TDO as well.

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Reversal of Triple-Negative Breast Cancer EMT by miR-200c Decreases Tryptophan Catabolism and a Program of Immunosuppression



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Abstract

Tryptophan-2,3-dioxygenase (TDO2), a rate-limiting enzyme in the tryptophan catabolism pathway, is induced in triple-negative breast cancer (TNBC) by inflammatory signals and anchorage-independent conditions. TNBCs express extremely low levels of the miR-200 family compared with estrogen receptor-positive (ER⁺) breast cancer. In normal epithelial cells and ER⁺ breast cancers and cell lines, high levels of the family member miR-200c serve to target and repress genes involved in epithelial-to-mesenchymal transition (EMT). To identify mechanism(s) that permit TNBC to express TDO2 and other proteins not expressed in the more well-differentiated ER⁺ breast cancers, miRNA-200c was restored in TNBC cell lines. The data demonstrate that miR-200c targeted TDO2 directly resulting in reduced production

of the immunosuppressive metabolite kynurenine. Furthermore, in addition to reversing a classic EMT signature, miR-200c repressed many genes encoding immunosuppressive factors including *CD274/CD273*, *HMOX-1*, and *GDF15*. Restoration of miR-200c revealed a mechanism, whereby TNBC hijacks a gene expression program reminiscent of that used by trophoblasts to suppress the maternal immune system to ensure fetal tolerance during pregnancy.

Implications: Knowledge of the regulation of tumor-derived immunosuppressive factors will facilitate development of novel therapeutic strategies that complement current immunotherapy to reduce mortality for patients with TNBC.

Introduction

Triple-negative breast cancer (TNBC), which lacks estrogen and progesterone receptors (ER and PR) and amplification of the growth factor receptor HER2 (human EGFR 2), recurs more often and more rapidly than other subtypes, with a peak risk of recurrence at 1 to 3 years postdiagnosis (1). If metastatic at the time of initial diagnosis, TNBC has a median survival of 13 months (2, 3). This high metastatic potential can be facilitated by an oncogenic epithelial-to-mesenchymal transition (EMT) that enhances anchorage independence and invasiveness. Partial EMT or mixed populations potentiate metastasis in breast cancer preclinical models (4) and circulating tumor cells isolated from patients with breast cancer were enriched for mes-

enchymal markers upon chemoresistance (5). Estrogen receptor-positive (ER⁺) breast cancer cell lines maintain epithelial markers and undergo significant programmed cell death termed "anoikis" under anchorage-independent conditions (6). In contrast, TNBC lines aberrantly express mesenchymal and neuronal proteins that provide survival signals (7, 8) and metabolic changes (9, 10) including increased tryptophan catabolism (11).

Increasing evidence supports a role for tryptophan catabolism in tumor progression, whereby the tryptophan catabolite kynurenine (Kyn) facilitates anchorage-independent survival by binding to and activating the aryl hydrocarbon receptor (AhR; refs. 11–13). Although the enzymes indoleamine 2,3-dioxygenase 1 (IDO1) and IDO2 can mediate the rate-limiting step of tryptophan catabolism, we reported that in TNBC the increased intracellular and secreted levels of Kyn critical for anchorage-independent survival are mediated by tryptophan-2,3-dioxygenase (TDO2) activity (11). Kyn can also exert its effects in a paracrine fashion, by binding to AhR in CD8⁺ T cells to suppress antitumor activity (12, 14, 15), and by expanding the regulatory T cell population to exhaust the antitumor response (14).

Members of the miR-200 family are high in normal epithelial cells and the more well-differentiated ER⁺ breast cancers, but extremely low in TNBC, and miR-200c is the most differentially expressed between human ER⁺ versus TNBC cell lines (16–18). Considered the "guardians of the epithelial phenotype," this miRNA family suppresses EMT by direct targeting of Zinc Finger

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E-Box Binding Homeobox 1 and 2 (ZEB1/2) and other transcriptional repressors of E-cadherin (19, 20), as well as numerous mesenchymal, neuronal, and embryonic stem cell genes (21, 22). Loss or silencing of this miRNA family in TNBC permits aberrant production of nonepithelial proteins that facilitate multiple steps in the metastatic cascade.

Here we report that restoration of miR-200c to TNBC cell lines not only potently reversed a "pan-EMT gene signature" derived from multiple types of carcinomas, but also significantly decreased TDO2 and consequent production of Kyn. Furthermore, miR-200c repressed multiple genes encoding immunosuppressive factors known to be produced by early embryo cells called trophoblasts. Trophoblasts undergo a normal EMT that facilitates invasion into the uterus and suppresses the maternal immune system to ensure fetal tolerance. The commonalities between tumor cells and trophoblasts are hypothesized to be due to adaptations to similar selective pressures, including limited nutrients, oxygen, and the need to avoid immune recognition (23). Although the idea that "trophoblast mimicry" might facilitate tumor progression has been previously recognized (24, 25), a global mechanism permitting tumor cells to coopt this immunosuppressive program, had not been identified.

Materials and Methods

Cell lines

All cell lines were authenticated by Short Tandem Repeat DNA Profiling (Promega) at the University of Colorado Cancer Center (UCCC) Tissue Culture Core, and tested for *Mycoplasma* every 3 months. Only cells under five passages were used in this study. Hs578T and SUM159PT cells were purchased from the UCCC Tissue Culture Core and the rest directly from ATCC. SUM159PT (SUM159) cells were grown in Ham F-12 with 5% FBS, penicillin/streptomycin, hydrocortisone, insulin, HEPES, and L-glutamine supplementation. BT549 cells were cultured in RPMI 1640 medium with 10% FBS, penicillin/streptomycin, and insulin. MDA-MB-453 (MDA-453) were grown in DMEM with 10% FBS and penicillin/streptomycin. MDA-MB-231 (MDA-231) were grown in MEM with 5% FBS, HEPES, L-glutamine, nonessential amino acids, and insulin. MCF7 cells were obtained from Dr. Kate Horwitz at the University of Colorado Anschutz Medical Campus (Aurora, CO) and were grown in DMEM with 10% FBS and L-glutamine.

Reagents

IL1 β and TNF α were purchased from Affymetrix eBioscience and used at a final concentration of 10 ng/ μ L. Negative, scrambled control (SCR) or miR-200c mimics were purchased from Ambion and used at a final concentration of 50 nmol/L. miRNA mimic and luciferase reporter transfections were performed using either RNAi Max or Lipofectamine 3000 purchased from Thermo Fisher Scientific and experiments were conducted following the manufacturer's protocols. TDO2 cDNA plasmid (TDO2-OE) and corresponding empty plasmid control vector (EV) control were obtained from Sino Biological (catalog nos. HG13215-UT and CV011).

Inducible system for miR-200c expression

BT549 cells were transduced with the doxycycline-inducible lentiviral vector pTripZ (Dharmacon) encoding the precursor

sequence for miR-200c (pTripZ-200c). Control cells were transduced with pTripZ empty vector (pTripZ-EV, pooled population). Stable expression was selected using 1 μ g/mL puromycin, and multiple pTripZ-200c clones were tested to identify which had low/absent background expression of miR-200c and at least 500-fold expression of miR-200c in the presence of 1 μ g/mL doxycycline inducer.

Sample preparation/UHPLC-MS analysis of cell lines

Cells were counted, pelleted at $1,500 \times g$ at 4°C, and stored at –80°C until analysis. Prior to LC-MS analysis, samples were placed on ice and resuspended with methanol:acetonitrile:water (5:3:2, vol/vol) at a concentration of 2×10^6 cells per mL. Media samples were extracted with the same solution at a dilution of 1:25 (vol/vol). Suspensions were vortexed continuously for 30 minutes at 4°C. Insoluble material was removed by centrifugation at $10,000 \times g$ for 10 minutes at 4°C and supernatants were isolated for metabolomics analysis by UHPLC-MS. Briefly, the analytic platform employs a Vanquish UHPLC system coupled online to a Q Exactive Mass Spectrometer (Thermo Fisher Scientific). Samples were resolved over a Kinetex C18 column, 2.1×150 mm, 1.7 μ m particle size (Phenomenex) equipped with a guard column (SecurityGuard Ultracartidge – UHPLC C18 for 2.1 mm ID Columns – AJO-8782 – Phenomenex) using an aqueous phase (A) of water and 0.1% formic acid and a mobile phase (B) of acetonitrile and 0.1% formic acid. Samples were eluted from the column using either an isocratic elution of 5% B flowed at 250 μ L per minute and 25°C or a gradient from 5% to 95% B over 1 minute, followed by an isocratic hold at 95% B for 2 minutes, flowed at 400 μ L per minute and 30°C. The Q Exactive mass spectrometer (Thermo Fisher Scientific) was operated independently in positive or negative ion mode, scanning in Full MS mode (2 μ scans) from 60 to 900 m/z at 70,000 resolution, with 4 kV spray voltage, 15 sheath gas, and 5 auxiliary gas. Calibration was performed prior to analysis using the Pierce Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Acquired data were then converted from .raw to .mzXML file format using Mass Matrix. Metabolite assignments, isotopologue distributions, and correction for expected natural abundances of deuterium, ^{13}C , and ^{15}N isotopes were performed using MAVEN. Graphs, heatmaps, and statistical analyses (either *t* test or ANOVA), metabolic pathway analysis, PLS-DA, and hierarchical clustering were performed using the MetaboAnalyst 3.0 package (www.metaboanalyst.com). Hierarchical clustering analysis was also performed through the software GENE-E (Broad Institute, Cambridge, MA). XY graphs were plotted through GraphPad Prism 5.0 (GraphPad Software Inc.).

qRT-PCR

Total RNA was isolated using TRIzol RNA Isolation (Qiagen) according to the manufacturer's instructions. cDNA was synthesized with the qScript cDNA SuperMix (QuantaBio). qRT-PCR was performed in an ABI 7600 FAST Thermal Cycler. SYBR Green quantitative gene expression analysis was performed using Absolute Blue qRT-PCR SYBR Green Low ROX Mix (Thermo Fisher Scientific) and the following primers: TDO2 forward, 5'-CGGTGGTTCCTCAGGCTATC-3'; reverse, 5'-CTTCGGTATCCAGTGTCTGGG-3'; IL6 forward, 5'-AAGCCA-GAGCTGTGCAGATGA-3'; reverse, 5'-AACAAACATCTGAGGT-GCCCA; HMOX-1 forward, 5'-CAGGCAGAGAATGCTGAGTTC-

3'; reverse, *GDF-15* forward, 5'-CTCAGGACGCTGAATGGC-TCT-3'; reverse 5'-GGGTCTTGCAAGGCTGAGCTG-3'; *PD-L1* forward, 5'-TATGGTGGTGGCGACTACAA-3'; reverse, 5'-TGGC-TCCCAGAATTACCAAG-3'; *ZEB1* forward, 5'-TCCATGCTTAA-GAGCGTAGCT-3'; reverse, 5'-ACCGTAGTTGAGTAGGTGTA-TGCCA-3'; *GAPDH*, forward 5'-GTCAGTGGTGACCTGACCT-3'; reverse 5'-AGGGGTCTACATGGCAACTG-3'; β -actin: forward, CTGTCCACCTCCAGCAGATG; reverse, CGCAACTAA-GTCATAGTCCGC. Taqman quantitative gene expression analysis for *miR-200c* and *U6* was performed with primer-specific reverse transcriptase using the following TaqMan MicroRNA Assays (Applied Biosystems/Thermo Fisher Scientific): *miR-200c* assay ID #002300; *U6* assay ID #001973. All experiments were performed in biological triplicate.

Immunoblotting

Whole-cell protein extracts (50 μ g) were denatured, separated on SDS-PAGE gels, and transferred to polyvinylidene fluoride membranes. After blocking in 5% BSA in Tris-buffered saline-Tween, membranes were probed overnight at 4°C. The following antibodies were used in this study: TDO2 (Sigma, catalog no. HPA039611, 1:1,000), HMOX-1 (Cell Signaling Technology, catalog no. 5061, 1:1,000), ZEB1 (Sigma, catalog no. HPA027524, 1:2,000), α -tubulin (Sigma, catalog no. T5168, 1:10,000), and β -actin (Cell Signaling Technology, catalog no. 3700, 1:4,000). Polyclonal antisera directed against GDF-15 were generated using the C-terminus peptide KIDTGVSLQTYDDLLA (Zymed). Affinity-purified GDF-15 antibody was prepared using the SulfoLink coupling resin according to the manufacturer's instructions (Thermo Fisher Scientific). Following secondary antibody incubation, results were detected using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer). Densitometry quantifications were performed using ImageJ (NIH). First, all bands were normalized to their respective α -Tubulin or β -actin loading control. The values shown in figures are reported as a fold change compared with either untreated cells or cells transiently transfected with scrambled mimic (SCR) or doxycycline.

Flow cytometry

Single-cell suspensions of BT549 cells were prepared and stained using the anti PD-L1 (Clone MIH1, eBioscience) following the manufacturer's instructions, then fixed in 1% paraformaldehyde and stored at 4°C until analysis. Flow cytometry was conducted using the BD LSRFortessa Cell Analyzer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

Gene expression analysis

BT549-pTripZ-200c cells, plated in triplicate, were cultured in doxycycline for 48 hours. RNA was isolated using the RNeasy Plus Kit (Qiagen) and quality was measured on an Agilent 2100 Analyzer (Agilent Technologies) and RIN (RNA integrity number) ranged from 9.6 to 10.0. Gene expression was measured using the Affymetrix Human Transcriptome 2.0 array hybridization performed by the University of Colorado Cancer Center Genomics and Microarray core facility and was deposited in the Gene Expression Omnibus (GEO) database as GSE108271. Data were analyzed by one-way ANOVA performed at the gene and probe (exon) levels using Partek Genomics Suite software (Partek). Microarray data were normalized by Robust Multiarray Average method using Affymetrix Power Tools. Multiple probe sets for the same gene were collapsed using average expression. Genes with

FDR < 10% and fold change > 1.2 were selected as differentially expressed between the two groups. Pathway analysis was performed using Gene Set Enrichment Analysis (GSEA) software and KEGG gene sets. Gene sets with $P < 0.05$ (after 1000 gene set permutations) were deemed to be enriched in each group.

Pan-carcinoma EMT signature analysis

To determine the EMT states of the untreated and doxycycline-treated BT549 cells, our gene array results were compared with the published pan-carcinoma EMT signatures from Mak and colleagues (26). The "pan-carcinoma EMT signature" genes were extracted from the normalized gene expression data of the untreated and doxycycline-treated BT549 cells. Unsupervised clustering was performed using CLUSTER 3.0 with Spearman correlation with average linkage. Cluster was visualized using Java TreeView (SourceForge).

Cloning of TDO2 3'UTR and site-directed mutagenesis

The 3'UTR of TDO2 was amplified from the BAC plasmid RP11-401G24. Primers were designed to amplify the 3'UTR of TDO2 and to additionally add restriction enzyme cut sites for *Sac1* (5' end) and *Xho1* (3' end): *Xho1*-TDO2: AGACCGCTCGA-GAATCGTCTGCAAATCTATG; *Sall*-TDO2: GAACGCGTCGAC-TACAGGGAGAAAGATTAATAC. Once amplified, the insert was cloned into the pmiR-GLO vector (Promega) using the corresponding restriction enzyme cut sites. The pmiR-GLO vector containing the 3'UTR of TDO2 was confirmed by gel electrophoresis and its sequence confirmed by the DNA Sequencing Core at the University of Colorado (Aurora, CO).

Luciferase reporter activity

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 and centrifuged to eliminate cell debris. The supernatant was used immediately or diluted with 1 $\times$ passive lysis buffer for determination of luciferase activity. For analysis of the activity of miR-200c mimic on the wild-type or mutated TDO2 3'UTR, both were cloned into the pmiR-Glo vector and luciferase activity was normalized to the *Renilla* signal to control for differences in transfection efficiency.

Measurement of anchorage-independent growth

Soft-agar assays were performed in 0.5% bottom and 0.25% top-layer agar (Difco Agar Noble; BD Biosciences). Cells were embedded in the top-layer agar and media refreshed every 4 days.

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

Restoration of miR-200c to TNBC cell lines leads to widespread reversal of EMT and an immunosuppressive signature that includes TDO2

BT549 breast cancer cells engineered to express miR-200c in a doxycycline-inducible manner were treated with or without doxycycline for 48 hours. RNA was collected, gene expression

analysis performed, and unsupervised hierarchical clustering of the top differentially expressed genes (FDR < 10% and fold change > 1.2) is shown in heatmap form (Fig. 1A). Analysis of miR-200c and known target *ZEB1* from BT549-TripZ-EV and BT549-TripZ-200c cells treated with or without doxycycline showed significantly increased miR-200c and a reduction in *ZEB1* in doxycycline-treated BT549-TripZ-200c cells exclusively (Fig. 1B). Western blot analysis confirmed decreased *ZEB1* and increased E-cadherin proteins in doxycycline-treated BT549-TripZ-200c, but not BT549-TripZ-EV cells (Fig. 1C). GSEA revealed alterations in a number of pathways involved in immune modulation upon induction of miR-200c, including tryptophan metabolism, allograft rejection, complement and coagulation pathways, and cytokine/cytokine receptor signaling (Fig. 1D; Supplementary Fig. S2; Supplementary Table S1). The list of altered genes involved in these immune-related pathways is available in Supplementary Table S2. For select genes encoding proteins with immunosuppressive functions, qRT-PCR was performed from RNA isolated from an independent set of BT549-TripZ-200c cells treated with or without doxycycline. This experiment demonstrated significantly decreased levels of *TDO2*, programmed death-ligand 1 (*PD-L1/CD274*), heme oxygenase 1 (*HMOX-1*), and growth differentiation factor 15 (*GDF-15*) in the presence of miR-200c (Fig. 1E). Although *GDF-15* was decreased by 1.7-fold, it does not appear in the heatmap because its *P* value fell slightly below the cutoff of FDR < 10% at FDR = 12%, but we followed up on its regulation because of its known immunomodulatory functions. Comparison of genes altered by induction of miR-200c in BT549-TripZ-200c cells to a "pan-carcinoma EMT signature" derived from multiple types of carcinomas (26) revealed that induction of miR-200c increased 60% (15/25) of the epithelial signature and decreased 80% (41/51) of the mesenchymal signature (Supplementary Fig. S1A).

Restoration of miR-200c in an immunocompetent mouse model of triple-negative mammary carcinoma led to decreased metastatic outgrowth in the lungs (GSE62230; ref. 27). Although the immune system was not examined in that study, genes altered by restoration of miR-200c to human TNBC cells compared with those altered by miR-200c in the mouse mammary carcinoma found a significant number of overlapping genes, including *TDO2*, *HMOX-1*, *PD-L1/CD274*, and inhibitor of nuclear factor kappa B kinase subunit beta (*IKKB*) among others (Supplementary Fig. S1B).

miR-200c directly targets *TDO2* and decreases TNBC Kyn production

To further validate the decrease in *TDO2* upon miR-200c restoration observed in the initial gene expression findings, four TNBC cell lines (BT549, Hs578T, MDA-453, and SUM159) were transiently transfected with either miR-200c mimic or scrambled control mimic (SCR). Decreased *TDO2* expression was detected in all four cell lines following miR-200c transfection (Fig. 2A). Western blot analysis of whole-cell lysates showed decreased *TDO2* and *ZEB1* in three TNBC cell lines tested (Fig. 2B). A predicted miR-200bc/429-binding site was identified in the *TDO2* 3'UTR (nucleotide position 5'-395-400-3') using TargetScan. To test whether miR-200c directly binds to and targets *TDO2*, the entire 3'UTR of *TDO2* was inserted downstream of luciferase in the pmir-GLO reporter plasmid

(Fig. 2C). When this reporter was transfected into BT549 cells with miR-200c mimic or SCR, miR-200c mimic significantly decreased luciferase activity. This effect was reversed upon mutation of the predicted binding site for miR-200c in the *TDO2* 3'UTR (Fig. 2D). To test the impact of miR-200c and subsequent reduction in *TDO2* protein on tryptophan catabolism, Kyn levels were measured by UHPLC-MS following miR-200c induction in the BT549 TripZ doxycycline-inducible system. Intracellular and secreted Kyn levels were significantly reduced with doxycycline induction of miR-200c in the BT549 cells and the same effect was observed following addition of miR-200c mimic to the Hs578T and MDA-453 TNBC cell lines (Fig. 2E).

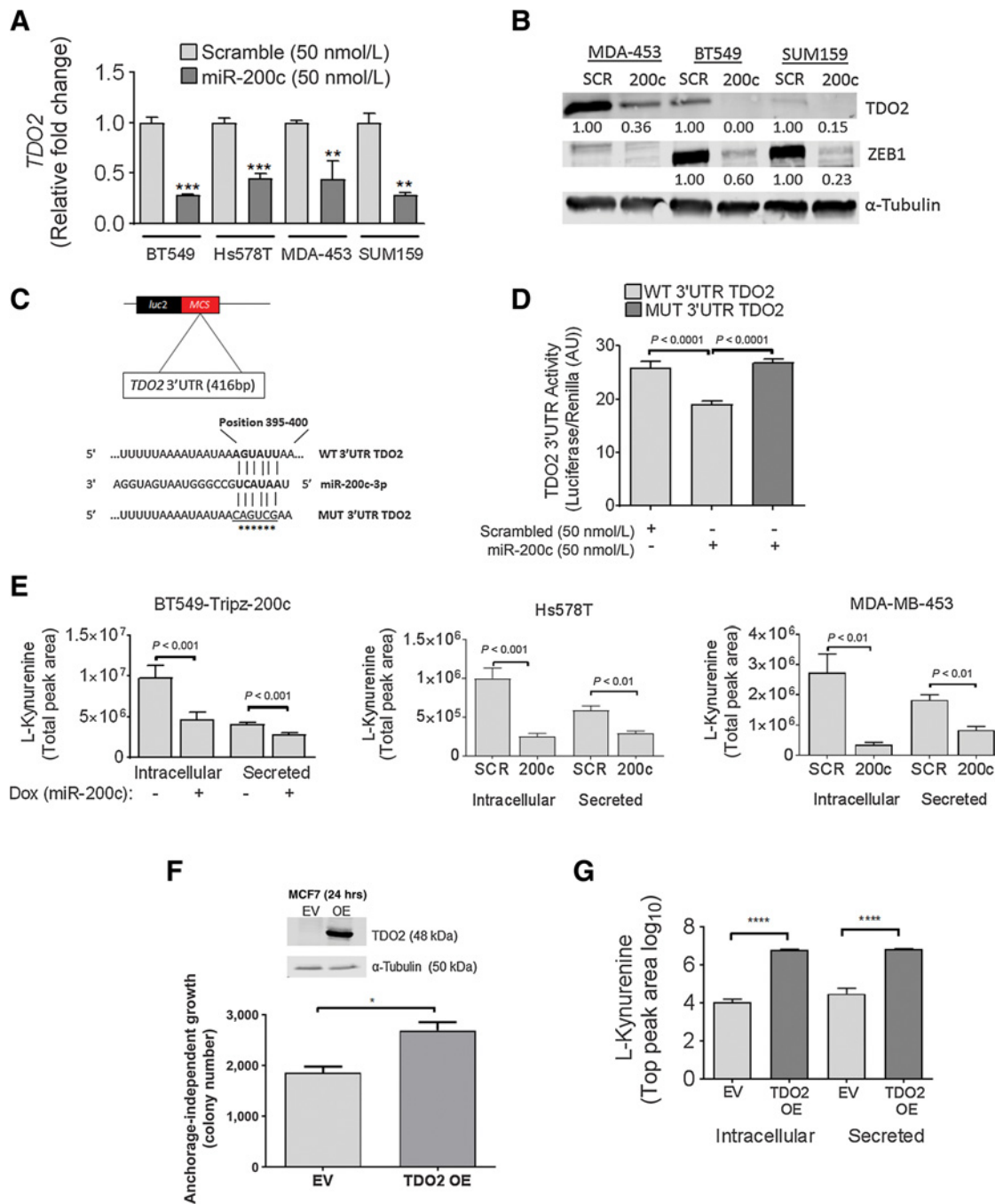
Exogenous *TDO2* in ER⁺ breast cancer cells increased anchorage-independent growth

TDO2 mRNA and protein are very low or undetectable in ER⁺ breast cancer cells and these cells do not survive well under anchorage-independent conditions compared with TNBC lines (11). To determine whether increased *TDO2* promotes anchorage-independent survival, MCF7 cells were transfected with either *TDO2* expression vector (*TDO2* OE) lacking the 3'UTR or empty vector (EV) for 24 hours and plated in soft agar. After 11 days, colonies were stained and quantified to assess anchorage-independent growth. MCF7-*TDO2* OE demonstrated significantly enhanced growth on soft agar compared with MCF7 EV cells (Fig. 2F). Western blot analysis of whole-cell lysate collected prior to growth on soft agar confirmed *TDO2* overexpression in the MCF7-*TDO2* OE cells as compared with MCF7-EV (Fig. 2F). Furthermore, to functionally confirm the activity of the exogenous *TDO2*, we confirmed by UHPLC-MS that MCF7-*TDO2* OE cells had increased intracellular and secreted Kyn levels compared with MCF7-EV cells (Fig. 2G).

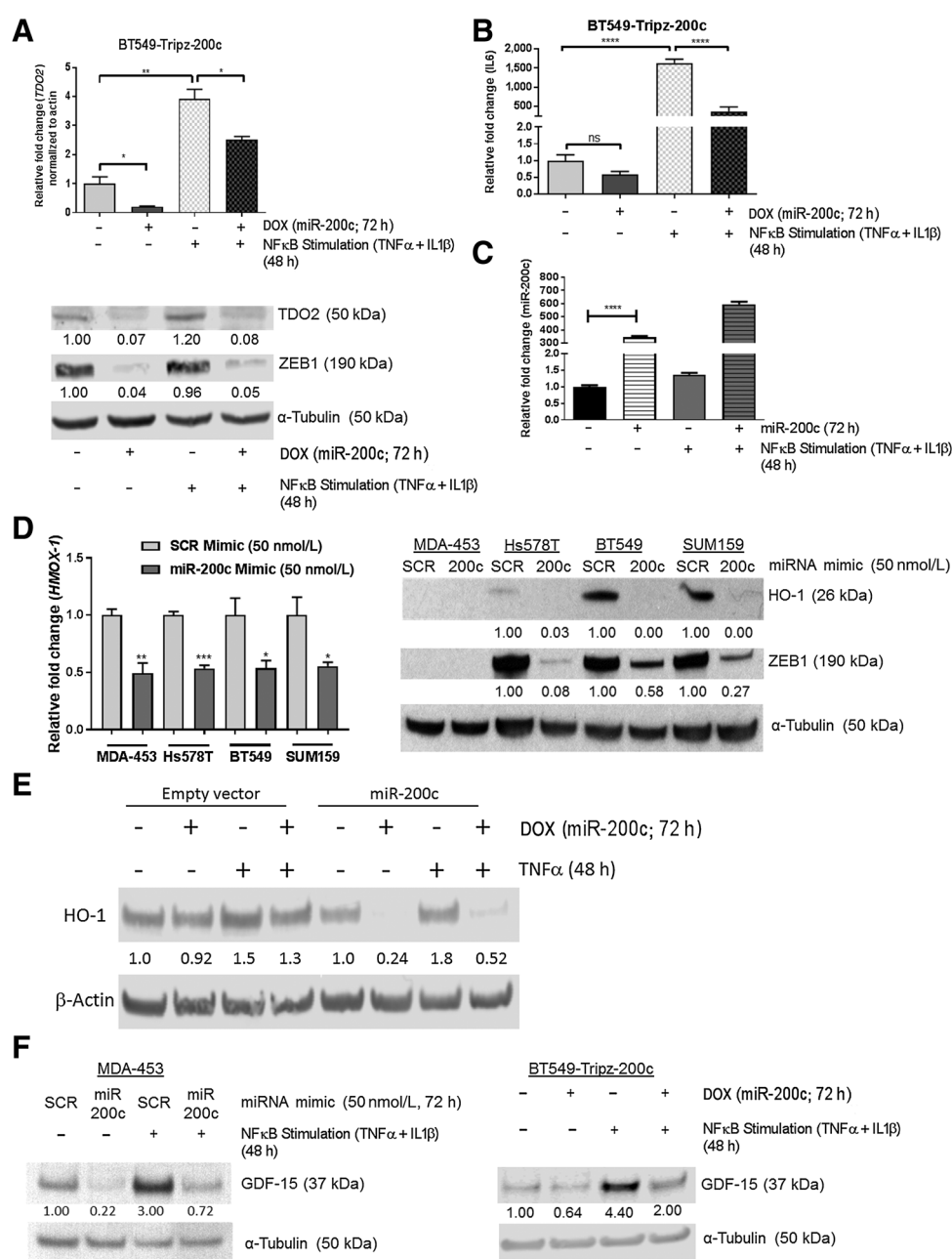
Restoration of miR-200c decreases *TDO2*, *HO-1*, and *GDF-15* even in the presence of inflammatory stimuli

TDO2 is significantly upregulated in TNBC cells in forced-suspension culture as a result of increased NFκB activity (11). Given these findings, we examined whether miR-200c would repress NFκB-mediated stimulation of *TDO2* expression. BT549-TripZ-200c-inducible cells were treated with or without doxycycline in the presence or absence of the NFκB-activating cytokines IL1β and TNFα. Both *TDO2* mRNA and protein were significantly upregulated following treatment with the NFκB-activating cytokines, but suppressed in the presence of doxycycline, indicating that miR-200c blocks *TDO2* expression even in the context of inflammatory stimuli (Fig. 3A). As a positive control, we examined *IL6*, a known NFκB target, which significantly increased following treatment with IL1β and TNFα (Fig. 3B). *IL6* was also decreased with restoration of miR-200c in the initial array results and this was confirmed in an independent experiment shown in Fig. 3B. Although miR-200c levels significantly increased following treatment with doxycycline, they were not significantly affected by IL1β and TNFα treatment (Fig. 3C).

We further investigated whether miR-200c decreased multiple other genes encoding immunomodulatory proteins (Fig. 1A; Supplementary Fig. S2). *HMOX-1*, which encodes heme oxygenase 1 (HO-1), was reduced by restoration of miR-200c in the microarray analysis (fold change -2.13; *P* = 9.40E-05).

**Figure 2.**

Restoration of miR-200c to TNBC decreases TDO2 by direct targeting of *TDO2* at a binding site in the 3'UTR, resulting in reduction of intracellular and secreted Kyn. **A**, Relative *TDO2* mRNA levels determined by qRT-PCR in multiple TNBC cell lines following transfection with either negative SCR control mimic or miR-200c for 48 hours. ***, $P < 0.001$, **, $P < 0.01$, unpaired *t* test. **B**, TDO2 and ZEB1 in multiple TNBC lines following transfection with negative control or miR-200c for 72 hours. **C**, Schematic of *TDO2* 3'UTR depicting predicted binding site of miR-200c at position 395-400 cloned into pmiR-Glo-luciferase vector with site-directed mutations indicated by asterisks. **D**, Luciferase activity of the WT *TDO2* 3'UTR or mutated *TDO2* 3'UTR containing pmiR-Glo following transfection of BT549 cells with either negative control or miR-200c for 48 hours. ****, $P < 0.0001$, one-way ANOVA analysis. **E**, Relative Kyn levels in BT549 cells as determined by UPLC-MS expressing a stable doxycycline-inducible lentiviral empty vector pTripZ vector or miR-200c-pTripZ vector after 72 hours of doxycycline induction (left). Relative Kyn levels measured in Hs578T and MDA-453 following transfection with SCR control or miR-200c mimic 72 hours prior (middle, right). **F**, MCF7 cells transfected with expression vector for *TDO2* (OE) or empty vector (EV) and immunoblot probed with TDO2-detecting antibody 24 hours after transfection (top). MCF7 cells with TDO2 OE or EV control were grown in soft agar and colony number determined after 11 days (bottom). **G**, Intracellular and secreted Kyn measured by UPLC-MS in MCF7-EV or MCF7-TDO2-OE cells after 72 hours. Data are shown on a log₁₀ scale.

**Figure 3.**

Restoration of miR-200c abrogates expression of immunomodulatory gene and protein expression in TNBC cells. **A**, Relative *TDO2* mRNA levels in BT549-TripZ-200c cells after 72 hours of no treatment, doxycycline (DOX), NFκB stimulation [TNFα, IL1β (10 ng/μL each)] for the last 48 hours, or the combination of doxycycline and NFκB stimulation (top). Relative *TDO2* levels were normalized to β-actin. ***, $P < 0.001$, **, $P < 0.01$ by unpaired *t* test. Immunoblot for TDO2 and ZEB1 with α-Tubulin as a loading control in BT549-TripZ-200c cells after 72 hours of no treatment, doxycycline, NFκB stimulation (TNFα and IL1β), or the combination of DOX and NFκB stimulation (bottom). Fold changes are indicated compared with vehicle treated after normalizing to loading control. **B**, *IL6* mRNA levels in BT549-TripZ-200c cells after 72 hours of no treatment, doxycycline, NFκB stimulation (TNFα, IL1β), or the combination of DOX and NFκB activation. Relative *IL6* levels were normalized to β-actin. ***, $P < 0.001$, **, $P < 0.01$ by unpaired *t* test. **C**, Relative *miR-200c* in BT549-TripZ-200c cells after 72 hours of no treatment, doxycycline alone, NFκB stimulation (TNFα, IL1β), or the combination of doxycycline plus NFκB activation. Relative *miR-200c* levels were normalized to *U6* snRNA. **D**, *HMOX-1* qRT-PCR in multiple TNBC cell lines following transient transfection with either negative control scrambled (SCR) or miR-200c mimic after 48 hours (left) and immunoblot for HO-1 (encoded by *HMOX-1*) in TNBC lines following transient transfection with SCR or miR-200c mimic for 72 hours (right). Fold changes were calculated over SCR after normalizing to the α-Tubulin loading control. **E**, Immunoblot for HO-1 protein in BT549-Tripz-EV cells as compared with BT549-Tripz-200c cells treated with or without DOX (+/-; 72 hours) with or without TNFα for the last 48 hours. **F**, Immunoblot for GDF-15 in MDA-453 cells following transfection with SCR control or miR-200c mimic for 72 hours with or without NFκB stimulation for the last 48 hours (left) and in BT549-TripZ-200c cells after vehicle versus doxycycline for 72 hours with or without NFκB stimulation for the last 48 hours (right). Fold change was calculated over vehicle-treated cells after normalization to α-Tubulin loading control.

Following transient transfection of BT549, Hs578T, MDA-453, and SUM159 TNBC lines with miR-200c mimic, *HMOX-1* was significantly reduced in all lines (Fig. 3D, left). HO-1 protein (encoded by *HMOX-1*) was also decreased by miR-200c (Fig. 3D, right). Because TNF α increased HO-1 protein levels, but IL1 β decreased them (Supplementary Fig. S3), we examined HO-1 in empty vector containing BT549-TripZ-EV cells versus miR-200c containing BT549-TripZ-200c cells treated with or without doxycycline, then plus or minus TNF α . It was clear that even in the face of TNF α stimulation, miR-200c suppressed production of HO-1 (Fig. 3E).

GDF-15, which encodes an anti-inflammatory cytokine also known as placental TGF- β (PTGFB) due to its high level of expression in placenta, decreased in BT549-TripZ-200c cells following induction of miR-200c in the array analysis (fold change -1.7); however, the *P* value fell slightly below the cutoff of $P < 0.1$, with $P = 0.12$. We examined whether NF κ B stimulation affected GDF-15 levels and whether miR-200c would limit this process. BT549-TripZ-200c-inducible cells were treated with or without doxycycline in the presence or absence of the NF κ B-stimulating cytokines IL1 β and TNF α for 48 hours. GDF-15 protein was upregulated by 4.4-fold following cytokine treatment, but the effect was suppressed in the presence of doxycycline induction of miR-200c (Fig. 3F, right). The same was true in MDA-453 cells, in which the NF κ B-activating cytokines significantly upregulated GDF-15, but addition of miR-200c mimic repressed both GDF-15 baseline and cytokine-induced levels (Fig. 3F, left).

Restoration of miR-200c decreases expression of PD-L1/2

PD-L1 was recently identified as a direct target of miR-200c in lung cancer and MCF7 breast cancer cells (28, 29). Because *PD-L1* was significantly decreased in BT549-200c cells following doxycycline induction in the array analysis (Fig. 1A), we treated BT549-TripZ-200c cells with IFN γ to induce *PD-L1* expression, and confirmed that miR-200c significantly reduced the IFN γ -mediated upregulation of *PD-L1* (Fig. 4A). In addition, IFN γ -induced *PD-L2* expression was significantly reduced in the presence of miR-200c (Fig. 4B). Reduction in PD-L1 ligand following restoration of miR-200c was confirmed by flow cytometry (Fig. 4C). Figure 4D illustrates a model of how miR-200c suppression of TDO2-dependent tryptophan catabolism and resulting Kyn production, along with suppression of GDF-15 and HO-1, might affect T cells in a noncontact-dependent fashion in addition to reducing the contact-dependent immunosuppressors, PD-L1 and 2.

Discussion

Here we report a novel mechanism of regulation of tumor tryptophan catabolism, involving post transcriptional regulation by miR-200c. The miR-200 family is well-described as a critical determinant of the epithelial phenotype that directly targets and suppresses transcriptional repressors of E-cadherin such as ZEB1 and ZEB2, Twist, and Snail, to mediate normal EMT that facilitates rearrangement of epithelial cells during development, as well as oncogenic EMT in carcinomas to facilitate metastasis (19, 20). Many mesenchymal and neuronal genes involved in motility, invasiveness, anoikis resistance, chemoresistance, and stem cell-like properties such as tropomyosin-related kinase B (*TrkB*), neurotrophin 3 (*NTF3*), *moesin*,

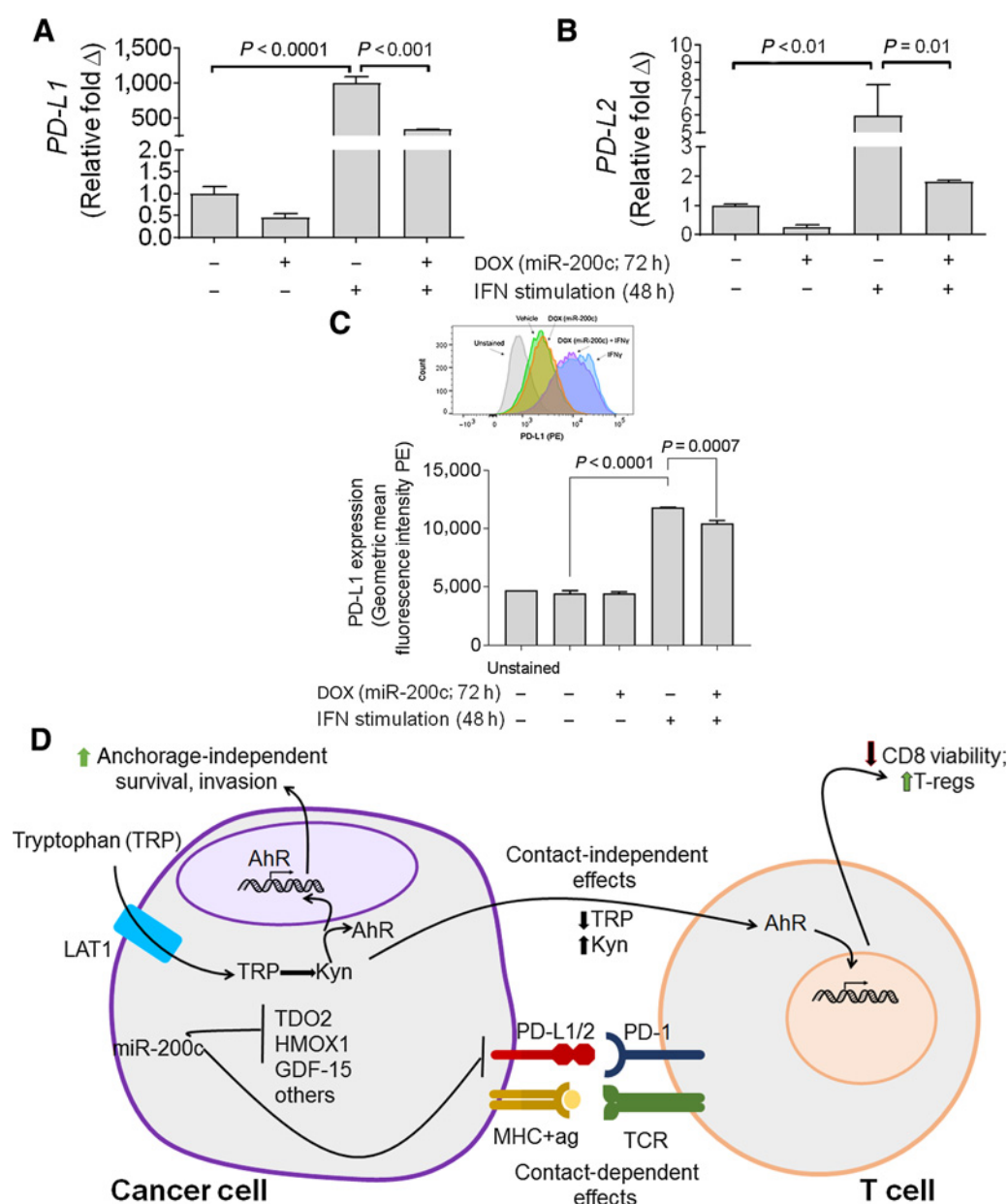
tubulin beta 3 class III (*TUBB3*), and suppressor Of zeste 12 protein homolog (*SUZ12*) are targeted by miR-200c (21, 30). In many dedifferentiated, aggressive carcinomas, including TNBC, miR-200 family members are often lost or repressed by microdeletions or silenced via methylation (31, 32). Here, restoration of miR-200c to TNBC cells repressed ZEB1, restored E-cadherin, profoundly reversed a patient-derived, pan-cancer EMT signature (26), and significantly repressed genes encoding immunosuppressive factors. GSEA of genes reduced by induction of miR-200c in TNBC revealed a change in a number of pathways involved in immunomodulation including allograft rejection, complement and coagulation pathways, and tryptophan metabolism.

Analysis of genes increased by induction of miR-200c demonstrated the expected gain of epithelial characteristics, but also gave indications of increased glycolysis and oxidative phosphorylation. Although this alteration was unexpected, a recent study of the metabolic properties of an immortalized breast epithelial cell line and its EMT counterpart also found glycolysis and oxidative phosphorylation to be associated with the epithelial phenotype, while amino acid anaplerosis and fatty acid oxidation drove the mesenchymal phenotype (33). Interestingly, the tryptophan transporter LAT1 was deemed "essential for mesenchymal cell survival (33)."

Multiple clinical trials evaluating inhibitors of IDO activity such as Indoximod (D-1-methyl-tryptophan, NewLink Genetics) and INCB024360 (Incyte) either have already been conducted or are underway. However, these compounds do not inhibit TDO2, which evolved separately to metabolize tryptophan (34, 35). Data presented here, in our previous report, and by others, suggest that TDO2 may be relevant in TNBC and other types of cancer (11–13). Thus, new drugs that inhibit both IDO and TDO2 may be more effective in some cancer types including TNBC. TDO2-mediated production of Kyn supports tumor cell anchorage-independent growth and invasion in an autocrine manner by binding to and activating AhR (11–13). Indeed AhR was recently linked to inflammation and tryptophan metabolism in breast cancer (36). Kyn can also act in a paracrine fashion by binding AhR in T cells to suppress T cell-mediated tumor rejection (12, 37) via expansion of regulatory T cells (14) and direct effects on cytotoxic T cells (38). In addition to increased TDO2 upon NF κ B activation in TNBC (11), we now report posttranscriptional control of TDO2 by miR-200c, explaining why this enzyme is expressed in TNBC, but not ER $^{+}$ lines, which have high levels of miR-200c (16). TDO2 is decreased as a consequence of direct targeting of the TDO2 3'UTR by miR-200c and Kyn production is consequently reduced. Conversely, transfection of TDO2 lacking the 3'UTR containing miR-200c-binding site into ER $^{+}$ cells increased expression of Kyn and growth on soft agar.

GSEA of genes altered by miR-200c showed a reduction in pathways involved in immunosuppression and tolerance. Interestingly, normal EMT enables extravillar cytotrophoblasts to invade into the maternal decidual stroma and produce immunosuppressive substances that ensure embryo maintenance/fetal tolerance during pregnancy (23). We demonstrate that restoration of miR-200c suppresses the ability of TNBC to make similar immunosuppressive factors. The miR-200 family typically serves as the "brakes" by which EMT is kept at bay in normal epithelium or ER $^{+}$ breast cancer. We propose that loss or silencing of the miR-200 family permits TNBC to undergo EMT and coopt this

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**Figure 4.**

miR-200c regulates PD-L1 and 2 and other immunosuppressive factors in TNBC. BT549-TripZ-200c cells were treated with doxycycline (DOX) to induce expression of miR-200c, or vehicle control. **A**, Relative *PD-L1* mRNA levels were determined by qRT-PCR in 24 hours after doxycycline treatment began, cells were treated with IFN γ (10 ng/mL) or vehicle. Cells were harvested 72 hours after the commencement of doxycycline treatment. ****, $P < 0.0001$, as determined by one-way ANOVA. **B**, Relative *PD-L2* mRNA levels were determined by qRT-PCR in BT549 cells expressing a stably transduced, doxycycline-inducible miR-200c vector were treated with doxycycline to induce expression of miR-200c, or vehicle control. Twenty-four hours after doxycycline treatment began, cells were treated with IFN γ (10 ng/mL) or vehicle. Cells were harvested 72 hours after doxycycline treatment. ****, $P < 0.0001$, as determined by one-way ANOVA. **C**, PD-L1 expression was measured by flow cytometry. BT549 cells expressing a stably transduced, doxycycline-inducible miR-200c vector were treated with doxycycline to induce expression of miR-200c, or vehicle control. Twenty-four hours after doxycycline treatment began, cells were treated with IFN γ (10 ng/mL) or vehicle. Cells were harvested 72 hours after the commencement of doxycycline treatment and stained for PD-L1. $n = 3$ samples, data shown are representative of two independent experiments. ***, $P < 0.001$, ****, $P < 0.0001$, as determined by unpaired t test. **D**, Model of factors repressed by restoration of miR-200c to TNBC and their published effects on T cells.

invasive/immunosuppressive program. In support of this theory, low levels of the miR-200 family member, miR-141, permit successful trophoblast uterine invasion and immunosuppression to facilitate successful tolerance of the fetus, whereas miR-141 is

abnormally high in preeclamptic placentae (when these processes fail; ref. 39).

Mellor and Munn described the role of tryptophan catabolism in the prevention of T cell-driven complement activation and

inflammation during pregnancy (40). Inhibition of IDO or TDO2-mediated tryptophan catabolism in pregnant mice induced fetal loss, supporting its role in maternal fetal tolerance (40). The immunosuppressive effects of both tryptophan depletion and increased tryptophan metabolites such as Kyn are well established (12, 14, 41). TDO2 also plays a role in endometrial decidualization where it suppresses the activation of decidual T cells to maintain pregnancy (42).

HO-1 is an anti-inflammatory, immunosuppressive, proangiogenic enzyme involved in suppression of CD8⁺ T cells through expansion of T-regulatory cells necessary for fetal tolerance during pregnancy (43). Gestational stress in mice led to lower HO-1, increased cytotoxic CD8⁺ T cells, and intrauterine growth restriction (44). Here, we show that restoration of miR-200c significantly decreased levels of *HMOX-1* (encoding HO-1) and HO-1 protein is overexpressed in breast cancer and predicts shorter overall survival (45). In clear cell renal cell carcinoma cells, miR-200c directly targets *HMOX-1* (46). In a mouse mammary carcinoma model, HO-1 inhibition reduced tumor growth to a degree comparable with PD-1 blockade (47). GDF-15 is highly expressed in placenta and decidual stromal cells in the uterus and has been found to promote tolerogenic dendritic cells during pregnancy (48). In malignant gliomas, depletion of GDF-15 enhanced natural killer cell-mediated clearance of tumors (49) and is also thought to help tumor cells evade immunosurveillance by macrophages (50). In the normal prostate, GDF-15 is inversely correlated with inflammation (51); however, circulating GDF-15 levels are elevated in patients with prostate cancer compared with men without prostate cancer. GDF-15 was significantly decreased by restoration of miR-200c to TNBC even in the face of NFκB stimulation.

Our data support recent reports that *PD-L1* (CD274) is targeted by miR-200c (28, 29), and we confirmed this finding at the protein level by flow cytometry. Restoration of miR-200c also reduced *PD-L2* (CD273) induction by IFNγ. Like *PD-L1*, *PD-L2* is expressed on both antigen-presenting cells and tumor cells and interacts with PD-1 on T cells to inhibit antitumor function (52). High tumor *PD-L2* indicated a better clinical response in patients treated with pembrolizumab (Keytruda), suggesting that PD-1 blockade may function through inhibition of both ligands (53).

Restoration of miR-200c decreased metastasis in an immunocompetent genetically engineered mouse model of claudin-low TNBC (27) and although changes in the immune response were not examined in that study, comparison of the data with the human TNBC cells studied here revealed overlap in the genes reduced upon induction of miR-200c, including *TDO2*, *HMOX-1*, *IKKB*, *PLCG1*, and *PD-L1* (Supplementary Fig. S1B). We observed that NFκB-stimulating cytokines upregulate *TDO2*, *HO-1*, and *GDF-15* protein in TNBC, but this induction by inflammatory stimuli is inhibited in the face of miR-200c as are baseline levels of these proteins. Although the ability of miR-200c to repress NFκB function may also be due to miR-200c affecting upstream regulators of this pathway such as *IKKB*, data presented here and by others indicate that *TDO2*, *HMOX-1* (46), *IL6* (54), and *PD-L1* (28, 29) are directly targeted by miR-200c. This insight into both the inflammation-mediated transcriptional and miRNA-mediated posttranscriptional regulation of specific immunosuppressive factors produced by TNBC cells may lead to new therapeutic strategies.

During pregnancy, infection can trigger preterm birth by activating the immune system, so dampening the immune response to infection and non-self antigens is advantageous in that setting. However, chronic inflammation may contribute to cancer by prompting epithelial cells to make immunosuppressive factors such as those described here, which would impede the antitumor immune response. Furthermore, we previously documented that NFκB activity increased in TNBC cells under anchorage-independent conditions (7, 11) and indeed *TDO2* and *HMOX-1* increased in TNBC under these conditions as well (8, 11). Thus, circulating tumor cells, which are able to resist the stress of anchorage-independent survival during dissemination, may also be particularly able to generate immunosuppressive metabolites that contribute to immune evasion. Interestingly, *HMOX-1* emerged in a recent synthetic lethal screen for genes supporting metastasis in a mouse mammary carcinoma model (55).

In a recent comparison of two isogenic mammary carcinoma cell lines, the more mesenchymal line expressed higher *PD-L1*, lower *MHC-1*, and contained increased T-regulatory cells and M2 macrophages in the tumor stroma (56); however, specific effectors or regulatory mechanisms were not identified. This study reveals multiple genes/proteins made by TNBC reminiscent of those used by trophoblasts to achieve fetal tolerance including *TDO2*, *PD-L1/2*, *HMOX-1*, and *GDF-15*. Although future studies are necessary to determine how to utilize this information to reverse tumor-induced tolerance and reactivate the immune system, some of these enzymes (*TDO2* and *HO-1*) and ligands (*GDF-15*) are targetable. The miR-200c family regulates the epigenetic modifier PcG protein Enhancer of Zeste Homolog 2 (*EZH2*), which affects the adaptive response to immunotherapies in melanoma (57). Although other lines of evidence have linked EMT and immunosuppression in cancer (26, 56, 58), our study functionally links miR-200c to an early embryonic program of immunosuppressive factors that ensure fetal tolerance. Therapeutic strategies targeting these tumor-derived noncontact-dependent immunosuppressive factors may increase the efficacy of checkpoint inhibitors in TNBC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Research

Reversal of Triple-Negative Breast Cancer EMT by miR-200c Decreases Tryptophan Catabolism and a Program of Immunosuppression

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Alterations in blood tryptophan and kynurenine levels have been examined in multiple human cancers (15–17). Lower serum tryptophan correlates with poor prognosis in melanoma, consistent with an increase in tryptophan catabolism (17). A small-scale study showed that plasma from postsurgical (lumpectomy or mastectomy) breast cancer patients has reduced tryptophan and elevated kynurenine compared with cancer-free controls, as measured by ultra-high pressure liquid chromatography (UHPLC), but importantly this study may not have been able to measure any potential effects of the primary tumor on systemic levels of these molecules (15). Another breast cancer study found relatively higher intratumoral kynurenine in ER[−] versus ER⁺ tumors (16). The issue of whether decreased tryptophan or increased kynurenine levels in breast cancer patient plasma reflect the activity of tumor tryptophan-catabolizing enzymes remains unresolved.

Here, we demonstrate that kynurenine increased primary human CD8 T-cell death and that conditioned media from anchorage-independent TNBC cells suppressed primary human CD8 T-cell function in a TDO-dependent manner, suggesting a mechanism of enhanced immune evasion during metastasis. Furthermore, we quantified tryptophan and kynurenine, as well as a series of tryptophan catabolites, in plasma from 77 presurgical breast cancer patients using UHPLC-MS and detected significant differences in plasma from women with breast cancer versus no breast cancer, ER⁺ versus negative tumors, and with increasing disease stage.

Materials and Methods

Cell culture

BT549 cells, purchased from ATCC in 2008 and authenticated prior to use by short tandem repeat DNA profiling (Promega), were grown in RPMI1640 medium with 10% FBS, penicillin/streptomycin, and insulin supplementation. Suspension culture was achieved using plates coated with 12 mg/mL poly (2-hydroxyethyl methacrylate) (Sigma-Aldrich) reconstituted in 95% ethanol. Ethanol evaporated overnight, and plates were washed with PBS prior to use.

Primary human T-cell isolation and activation

Blood was collected by venipuncture into tubes with heparin (BD Biosciences Vacutainer Systems) from donors with written informed consent under a University of Colorado Anschutz Medical Campus Colorado Institutional Review Board (COMIRB)-approved protocol. Lymphocytes were isolated by adding 10 mL of Ficoll-Paque PLUS (GE Healthcare) per 20 mL of blood diluted in Hank's balanced salt solution, then centrifuging at 2,000 rpm for 25 minutes without deceleration. CD8 T cells were resuspended in PBS containing 0.5% BSA and 2 mmol/L EDTA for selection using positive isolation kits (Dynabeads, Invitrogen). After isolation, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) according to manufacturer's instructions, then resuspended in RPMI medium containing 10% FBS, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and HEPES. T cells were activated in plates coated with 0.5 µg/mL of CD3 antibody (clone OKT3, eBioscience) and 1.0 µg/mL of soluble CD28 antibody (clone CD28.2, eBioscience) for 5 days. For intracellular staining, T cells were stimulated for 4 hours after the activation using phorbol-12-myristate-13-acetate (PMA) at 20 ng/mL, ionomycin at 1 µg/mL, and GolgiStop (BD Biosciences).

Cell staining and flow cytometry

T cells were stained with antibodies for CD3 (clone HIT3a, BioLegend) and CD8 (clone RPA-T8, BioLegend) at a 1:100 dilution and an intracellular stain for IFNγ (clone 4S.B3, eBioscience) at a 1:50 dilution. Intracellular staining was achieved using an intracellular fixation and permeabilization buffer set (eBioscience). Dead cells were detected by positivity for a viability dye according to manufacturer's instructions (Fixable Viability Dye BV421, eBioscience). Flow cytometry was conducted using an LSR II (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Inc).

TDO2 and AhR inhibition

The TDO2 inhibitor 680C91 (Sigma-Aldrich) or the AhR antagonist CH-223191 (TOCRIS Bioscience) prepared in dimethyl sulfoxide (DMSO) were used.

Breast cancer patient plasma

Blood was acquired as described above from presurgical breast cancer patients with written informed consent under a COMIRB- and HRPO-approved protocol (COMIRB 15-2225 and HRPO A-18613). The characteristics of the cohort are described in Supplementary Table S1.

UHPLC-MS–targeted metabolomic analysis of tryptophan catabolism

Tryptophan (¹⁵N₂) and kynurenine (ring-D₄, 3,3-D₂) were purchased from Cambridge Isotope Laboratories, Incorporated. To ensure linearity over 5 orders of magnitude, lysis buffer (LB), a 5:3:2 ratio of MeOH:acetonitrile:H₂O, and 0.1% formic acid aliquots were each spiked with varying ranges of heavy-labeled tryptophan and kynurenine (50 µmol/L, 5 µmol/L, 500 nmol/L, 50 nmol/L, 5 nmol/L, 500 pmol/L) and analyzed by ultra-high pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS). Plasma (20 µL) was extracted at 1:5 and 1:10 in LB containing 5 µmol/L heavy-labeled tryptophan and kynurenine as described previously, then diluted 1:5, 1:10 and 1:20 with H₂O and run on UHPLC-MS (18, 19). Sample extracts were analyzed via UHPLC-MS (Vanquish, Q Exactive – Thermo Fisher Scientific) using C18 reverse-phase chromatography and positive electrospray ionization (ESI; refs. 18, 20). A Kinetex C18 column, 2.1x150 mm, 1.7 µm particle size (Phenomenex) was used and equipped with a C18 guard column (Phenomenex). The method is a variant of previously published methods (21). Samples were resolved at 45°C with a gradient elution over 4 minutes, flowing at 450 µL/minute. Mobile phase A is 0.1% formic acid in water, mobile phase B is 0.1% formic acid in acetonitrile. 5% B and 95% A is held from 0.00 to 0.50 minutes. From 0.50 to 1.10 minutes, B increases to 95% B and 5% A. This condition is held from 1.10 to 2.75 minutes. From 2.75 to 3.00 minutes A decreases to the initial condition of 5% B and 95% A and is held from 3.00 to 4.00 minutes. The Q Exactive mass spectrometer was operated in positive ion mode using electrospray ionization, scanning in Full MS mode (1 µscan) from 100 to 1,500 m/z at 70,000 resolution, with 4 kV spray voltage, 45 sheath gas, 15 auxiliary gas. Calibration was performed prior to analysis using the Pierce Positive Ion Calibration Solution (Thermo Fisher Scientific). Metabolite assignments and heavy isotopologue detection for absolute quantitation against internal standards were determined against in-house standard libraries and KEGG database searches through Maven (22). Technical reproducibility was assessed by

monitoring internal heavy-labeled standard mixes as reported (18). Calculation of absolute quantification for measured metabolites was performed using the following formula: $[\text{light}] = (\text{abundance light}) / (\text{abundance heavy}) * [\text{heavy}]$ (dilution factor) where dilution factor is 10 for an extraction of 10 μL plasma in 90 μL lysis buffer.

Statistical analyses

Prism GraphPad Version 7.02 was used for all statistical analyses. All tests are two-sided with statistical significance set at $P < 0.05$. Statistical tests and sample sizes are described in the figure legends. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Results

Primary human CD8 T-cell response to kynurenine and cancer cell conditioned media

Infiltration into breast tumors by cytotoxic CD8 T cells correlates with improved prognosis in ER⁻ breast cancer (4, 23–25). To test the effect of kynurenine on primary human peripheral blood cytotoxic T cells, CD8⁺ cells isolated from peripheral blood of healthy human donors (Fig. 1A) were treated with increasing doses of kynurenine over a 5-day T-cell receptor (TCR) activation protocol. At 50 and 100 $\mu\text{mol/L}$ kynurenine, CD8 T-cell death increased as measured by a fixable viability dye (Fig. 1B), while CFSE analysis of live CD8 T cells indicated no change in proliferation (Fig. 1C), demonstrating a direct impact of kynurenine specifically on human CD8 T-cell viability. To test whether kynurenine acts through AhR to affect CD8 T-cell viability, CD8 T cells were treated with increasing doses of kynurenine with or without the AhR antagonist CH-223191 (26). Treatment with CH-223191 significantly reduced the cytotoxic effect of 50 $\mu\text{mol/L}$ kynurenine (Fig. 1D and E), indicating that kynurenine acts in part on CD8 T cells through AhR.

We previously reported that TNBC cells surviving under anchorage-independent culture conditions increased expression and activity of TDO, resulting in increased intracellular and secreted kynurenine (10). In the present study, we asked whether such anchorage-independent cells have enhanced immune evasion. BT549 TNBC cells were cultured in attached (ATT) or suspended (SUS) conditions for 24 or 48 hours. Primary human CD8 T cells were cultured in these conditioned media (CM) with CD3 and CD28 antibodies for 5 days. CD8 T cells cultured in SUS CM were less viable than those cultured in ATT CM (Fig. 2A). Because kynurenine decreased IFN γ mRNA in murine cytotoxic T cells (27), we tested whether CM from attached versus suspended TNBC cells would affect primary human CD8 T-cell production of IFN γ . Indeed, the SUS CM significantly reduced IFN γ production compared with ATT CM (Fig. 2B and C). To determine whether the effects of SUS CM were due to increased TDO activity, TNBC cells were plated in ATT or SUS conditions with or without TDO-specific inhibitor 680C91 for 48 hours. While SUS CM again increased CD8 T-cell death, this effect was significantly abrogated when SUS cells were treated with TDO inhibitor 680C91 (Fig. 2D), suggesting that TDO activity is at least partially responsible for the CM effect. However, treating the CD8 T cells with the AhR antagonist CH-223191 did not rescue the effect of SUS CM, suggesting additional effects of TNBC TDO activity on the CD8 T cells (Fig. 2E).

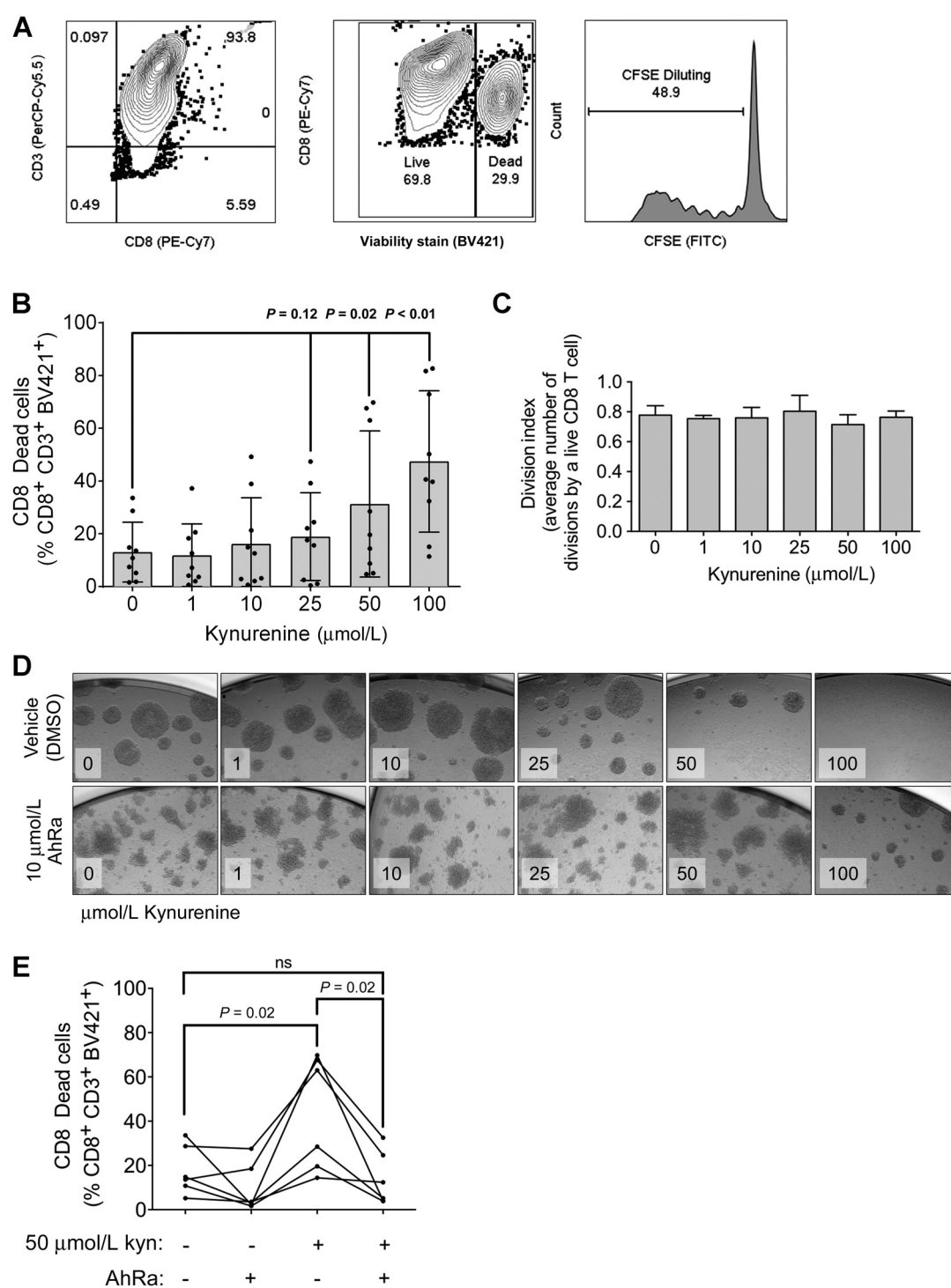
TDO2 RNA expression correlates with breast cancer outcomes, and tryptophan catabolism is altered in breast cancer

We previously reported that *TDO2* is significantly higher in breast cancer versus normal breast tissue and in ER⁻ versus ER⁺ disease, and that above-median *TDO2* correlated with poor overall survival in the Curtis and colleagues dataset (a dataset containing gene expression analysis from 2,000 breast tumors, extracted through the data-mining platform Oncomine; refs. 10, 28). In this study, we used the KM Plotter tool to investigate breast cancer outcomes associated with both *TDO2* and *IDO1* expression (29). High *TDO2* in primary breast cancer correlated with worse overall survival and reduced distant metastasis-free survival (Fig. 3A), while *IDO1* did not (Fig. 3B). Consistent with our findings that breast cancer cells have elevated tryptophan catabolism during anchorage independence, we also found, using the Curtis and colleagues dataset (28), that the gene encoding the amino acid transporter LAT1 (SLCA5) (which uptakes tryptophan and other large neutral amino acids from the microenvironment into the cell) was significantly elevated in breast cancer versus normal breast (Supplementary Fig. S1A), in ER⁻ versus ER⁺ disease (Supplementary Fig. S1B), and in higher grade breast cancer (Supplementary Fig. S1C). We also found using the Curtis and colleagues dataset and KM Plotter tool that above-median *LAT1* was associated with worse overall (Supplementary Fig. S1D and S1E) as well as distant metastasis-free (Supplementary Fig. S1F) survival as compared with below-median.

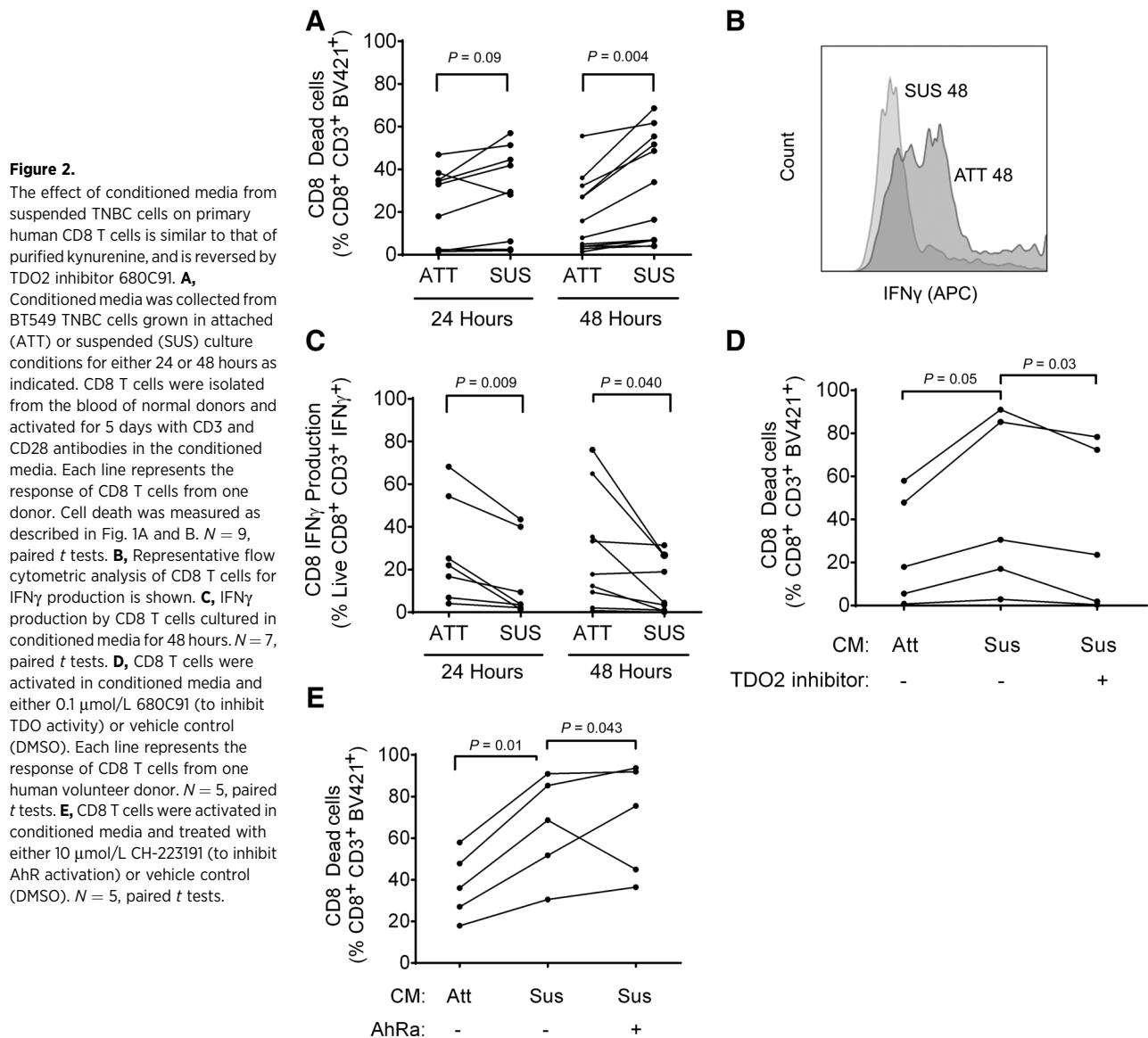
To further understand how tryptophan catabolism is altered in breast cancer subtypes, we measured plasma concentrations of tryptophan and its main catabolites, including kynurenine using UHPLC-MS (Supplementary Table S1). In the plasma from cancer-free "normal" women, the concentration of tryptophan ranged from 69.50 to 294.49 $\mu\text{mol/L}$, while in women with breast cancer, the range was 38.04–260.77 $\mu\text{mol/L}$. Meanwhile, the plasma concentration of kynurenine in normal women ranged from 3.46 to 13.61 $\mu\text{mol/L}$, and from 2.19 to 19.83 $\mu\text{mol/L}$ in women with breast cancer. Strikingly, there was a significant positive correlation between tryptophan and kynurenine both in plasma from normal women (Fig. 4A) and women with breast cancer (Fig. 4B). A comparison of the plasma concentrations of tryptophan and kynurenine in women with breast cancer versus normal controls demonstrated that women with breast cancer had significantly reduced tryptophan (mean = 141.39 $\mu\text{mol/L}$) compared with normal (mean = 173.32 $\mu\text{mol/L}$; Fig. 4C). Interestingly and surprisingly, the concentration of kynurenine was also significantly lower in the plasma of women with breast cancer (mean = 7.12 $\mu\text{mol/L}$) compared with normal controls (mean = 9.02 $\mu\text{mol/L}$; Fig. 4D).

Given previous reports that *TDO2* is significantly higher in ER⁻ versus ER⁺ disease, the plasma tryptophan and kynurenine concentrations in women with ER⁻ and ER⁺ breast cancer were compared next (10). There was no significant difference in either plasma tryptophan or kynurenine in women with ER⁺ and ER⁻ disease (Fig. 4C and D), though lower levels of tryptophan and anthranilate were observed in plasma from women with ER⁻, HER2⁻ disease (Supplementary Fig. S2). However, patients with ER⁻ breast cancer had significantly reduced plasma tryptophan compared with normal controls, while patients with ER⁺ breast cancer did not (Fig. 4C). Plasma kynurenine from both ER⁻ and ER⁺ breast cancer subsets was significantly lower than that from normal controls (Fig. 4D). Of note, these decreases were more marked in women with nonpregnancy-associated breast cancer

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**Figure 1.**

Increased kynurenine leads to primary human CD8 T-cell death, which is reversed by an AhR antagonist. **A**, Representative example of gating strategy for flow cytometric analysis: CD8 T cells were gated on the basis of double positivity for CD8 (PE-Cy7) and CD3 (PerCP-Cy5.5). Cell death was determined by positive staining for the fixable viability stain BV421. Proliferation was measured by CFSE dilution. **B**, Cells were activated with CD3 and CD28 antibodies for 5 days in the indicated concentration of kynurenine and CD8 T-cell death was determined. Each dot represents cells from one human donor. $N = 9$ donors, mean with SD, one-way ANOVA. **C**, Cells were treated with kynurenine as described in B and assayed for CD8 T-cell proliferation. $N = 9$ donors, one-way ANOVA. **D**, Representative images (10 $\times$ magnification) of CD8 T cells on activation day 5 that were treated with indicated concentrations of kynurenine and either 10 μ mol/L of the AhR antagonist CH-223191 (AhRa) or DMSO. **E**, CD8 T cells were activated, treated with 10 μ mol/L AhRa or vehicle control (DMSO), and assayed for cell death. $N = 6$, mean with SD, paired t tests.



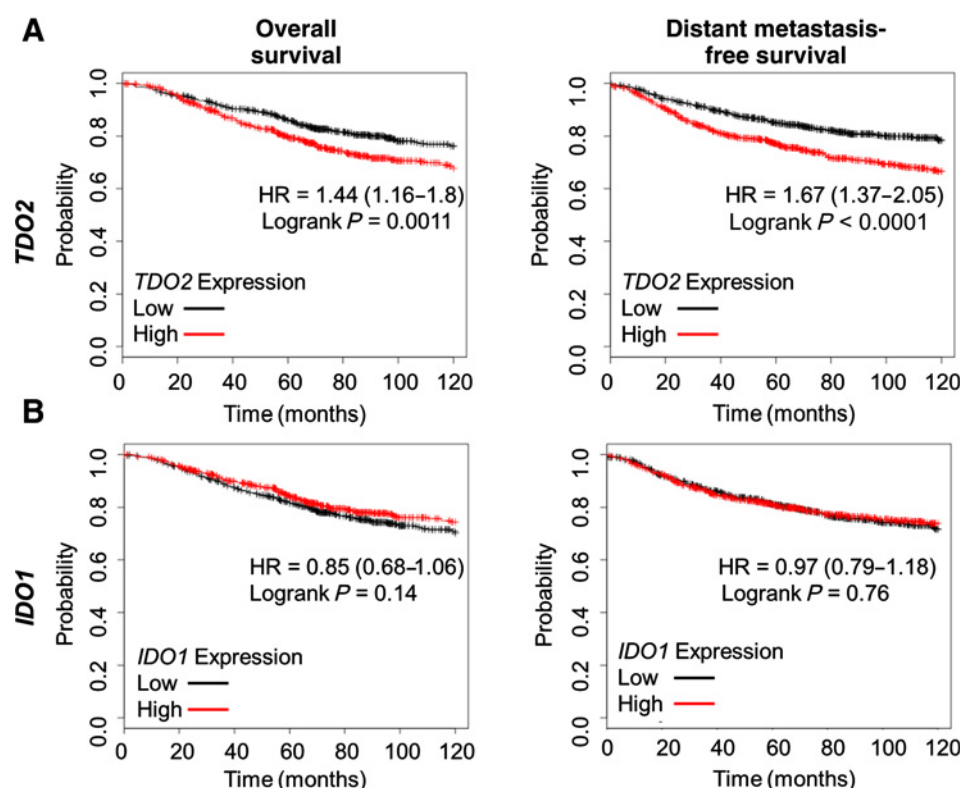
(PABC) in comparison with patients with PABC (where PABC is defined as a diagnosis with 5 years of pregnancy), with the highest relative levels of tryptophan (and its catabolites hydroxytryptophan, kynurenine, and kynurenic acid) detected in normal women who did not give birth within the past 5 years. Interestingly, women without a cancer diagnosis (normal), who had not given birth within the past 5 years, had intermediate levels of tryptophan and its catabolites in between the levels observed in normal women who had given birth within the past five years and PABC/non-PABC patients (Supplementary Fig. S3).

To examine whether either plasma tryptophan or kynurenine correlated with breast cancer aggressiveness, breast cancer stage at diagnosis was used. Both tryptophan (Fig. 4E) and kynurenine (Fig. 4F) were significantly lower in stage III/IV breast cancer patient plasma than in plasma from normal controls, while there was no difference in either tryptophan or kynurenine when comparing stage 0/I patients versus normal (Fig. 4E and F).

Interestingly, there was also a decrease in the kynurenine catabolite kynurenic acid in patients with stage III/IV breast cancer compared with patients with stage I/II disease (Supplementary Fig. S4).

Our finding that both tryptophan and kynurenine were reduced in the plasma of patients with breast cancer versus normal controls led us to investigate whether there were differences in nicotinamide, the final metabolite in the kynurenine pathway of tryptophan catabolism, between breast cancer patient and normal plasma. To do this, we calculated the ratio of tryptophan/nicotinamide in our dataset. We found that the tryptophan/nicotinamide ratio was significantly lower in the plasma from patients with breast cancer compared with normal controls, suggesting a potential overall increase in tryptophan catabolism not limited to the conversion of tryptophan to kynurenine (Fig. 4G). While the average relative abundance of nicotinamide was slightly elevated in breast cancer patient plasma compared

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**Figure 3.**

TDO2 expression correlates with breast cancer outcomes, while expression of the more commonly studied *IDO1* does not. **A**, Patients were split into *TDO2*-high (red, $n = 699$) or *TDO2*-low (black, $n = 703$) groups based on median *TDO2* gene expression. Overall survival (left) and distant metastasis-free survival (right) was plotted using KmPlot.com. **B**, Patients were split into *IDO1*-high (red, $n = 701$) or *IDO1*-low (black, $n = 701$) groups based on median gene expression, and overall survival and distant metastasis-free survival was plotted. $N = 1,402$. HRs are given with 95% confidence intervals, log-rank test.

with controls, this difference was not statistically significant (Fig. 4H). However, the ratio, which represents each individual's substrate (tryptophan) versus metabolite, is likely more meaningful given the person-to-person variability.

To examine whether tryptophan catabolism correlated with outcomes in this breast cancer dataset, we divided the patient data into tryptophan-low and tryptophan-high groups based on the median concentration of plasma tryptophan, and compared the progression-free survival of these groups, where progression was defined as local, regional, or distant recurrence, or patient death. While there was no significant difference in progression-free survival between these groups in patients with breast cancer with either ER⁺ (Fig. 5A) or ER⁻ (Fig. 5B) disease, there was a trend that did not reach statistical significance toward patients with lower than median plasma tryptophan having poorer outcomes (Fig. 5B), consistent with a negative impact of reduced tryptophan in ER⁻ disease.

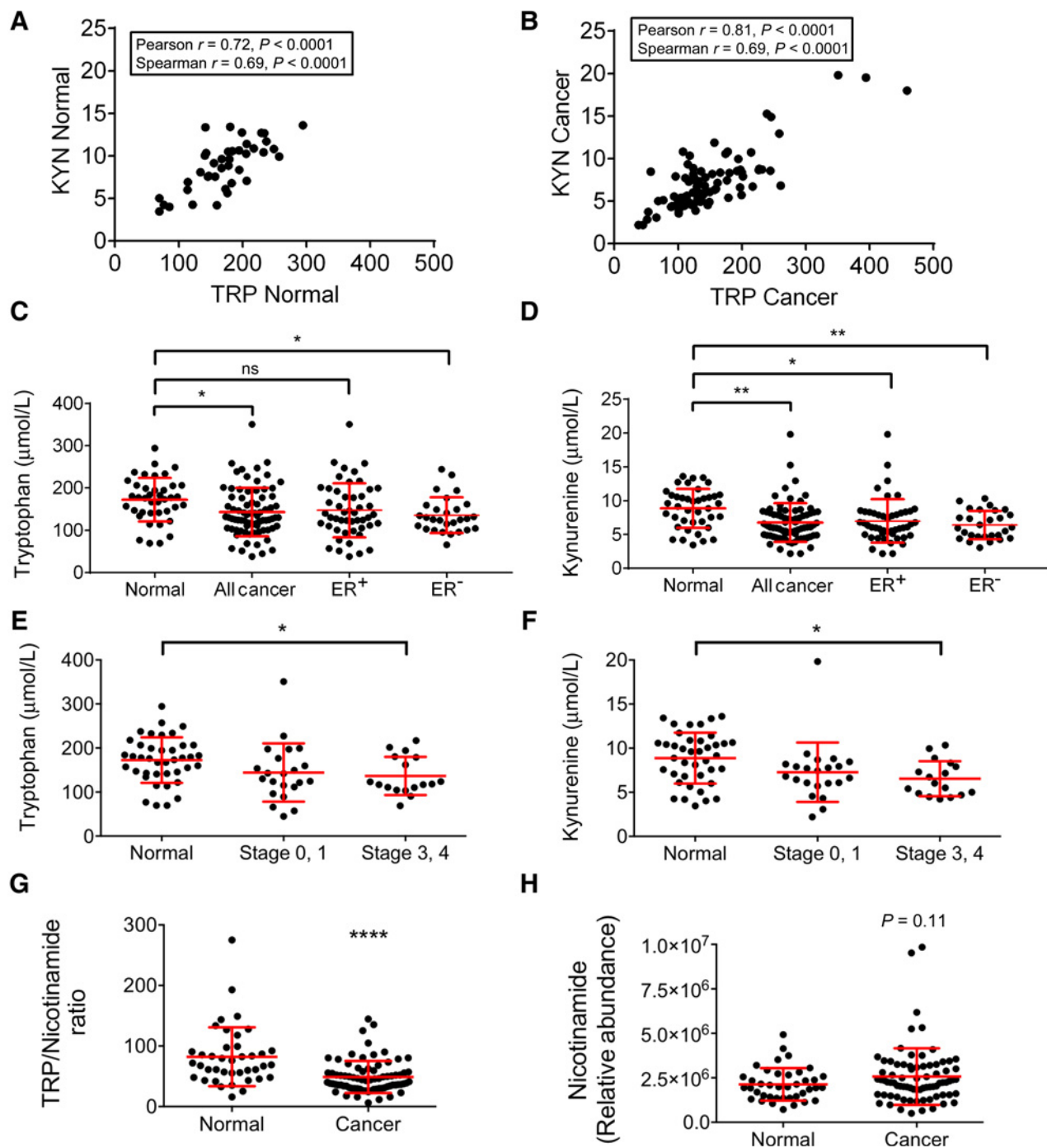
Discussion

While tryptophan catabolism is known to mediate tumor immune evasion, the impact of IDO or TDO activity on CD8 T cells was thought to occur through indirect suppression via regulatory T-cell activation. We demonstrate here that the direct impact of kynurenine on primary human CD8 T cells is to reduce viability in the setting of activation through the T-cell receptor. We previously reported that TDO activity increased in TNBC cell lines under anchorage-independent conditions, resulting in increased intracellular and secreted kynurenine (10). Critically, we show here that conditioned media from TNBC cells cultured under anchorage-independent conditions decreases the viability and

IFN γ production of primary human CD8 T cells, and that this effect is abrogated by the specific TDO inhibitor 680C91, indicating that anchorage-independent TNBC cells are highly immunosuppressive in part through TDO-mediated activity. A high ratio of cytotoxic CD8 T cells to Treg cells correlates with improved clinical outcomes in TNBC (30, 31). Thus, the power of TDO-positive metastatic TNBC cells to suppress CD8 T cells both directly and indirectly via the induction of Tregs (12) could critically contribute to disease progression. Interestingly, the concentration of kynurenine in the conditioned media was lower than the concentration of purified kynurenine that reduced CD8 T-cell viability, a phenomenon that could be explained by the milieu of other factors that are secreted by suspended TNBC cells that might have immunomodulatory effects (10).

Although inhibition of TDO activity in suspended TNBC cells abrogated the effects of conditioned media on the viability and function of CD8 cells, inhibition of the receptor for kynurenine, AhR, in the CD8 cells was not sufficient to mitigate this effect. One possible explanation for the differential effects of TDO as compared with AhR inhibition is that the effect of TDO activity on CD8 T cells is due to the combined effects of both tryptophan depletion and kynurenine secretion. Kynurenines combined with tryptophan depletion downregulate the TCR zeta-chain and decreases cytokine production in murine CD8 T cells (27), and IDO-mediated tryptophan depletion inhibits mTOR and PKC- θ activation to induce murine T-cell anergy and autophagy (32). AhR antagonism would interfere with kynurenine-mediated AhR activation, but would not abrogate tryptophan depletion.

To our knowledge, this study includes the largest metabolomic analysis of tryptophan and kynurenine in plasma from presurgery breast cancer patients, allowing assessment of the impact of an

**Figure 4.**

Tryptophan and kynurenine are reduced in breast cancer plasma. UHPLC/MS was used to measure tryptophan and kynurenine concentrations and nicotinamide abundance in the plasma from normal women and women with breast cancer. The concentrations of tryptophan and kynurenine correlate significantly and positively in both normal (**A**; $n = 40$) and breast cancer patient (**B**; $n = 77$) plasma by Pearson and Spearman tests. Comparison of tryptophan (**C**) and kynurenine (**D**) concentrations in plasma from normal women, all breast cancer patients in the cohort, and ER^+ versus ER^- breast cancer patients. Mean with SD, *, $P < 0.05$; **, $P < 0.01$, one-way ANOVA and Tukey multiple comparisons test. Comparison of tryptophan (**E**) and kynurenine (**F**) concentrations plotted by breast cancer stage is shown. Mean with SD, one-way ANOVA and Tukey multiple comparisons test. **G**, Comparison of the ratio of tryptophan (TRP) to nicotinamide between plasma from normal women and women with breast cancer. Mean with SD; ****, $P < 0.0001$, unpaired t test. **H**, Comparison of the relative abundance of nicotinamide in the plasma from normal and breast cancer patient plasma. Mean with SD, $P = 0.11$, unpaired t test.

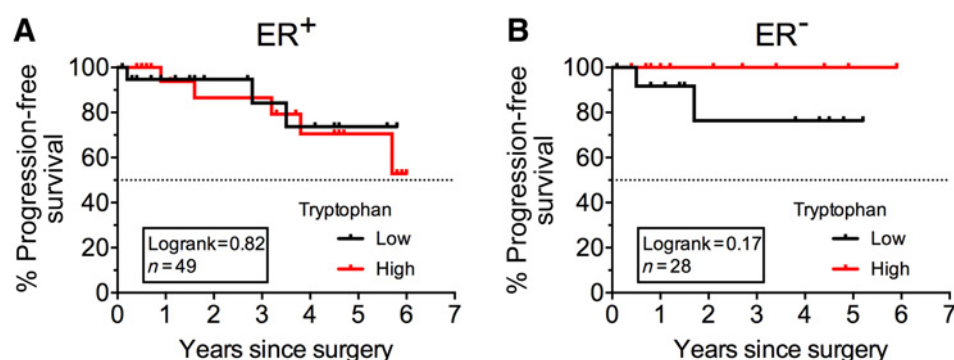


Figure 5. Breast cancer patient plasma tryptophan and progression-free survival. UHPLC/MS was used to measure tryptophan concentrations in the plasma from women with breast cancer. Kaplan-Meier curves plotting progression-free survival of breast cancer patients with ER⁺ disease ($n = 49$; **A**) or ER⁻ disease ($n = 28$; **B**) are shown, where progression was defined by local, regional, or distant recurrence, or patient death in the years after surgery, log-rank test.

existing breast tumor on systemic tryptophan and kynurenine levels. Given that others have reported higher kynurenine and lower tryptophan levels in breast cancer patients compared with healthy donors (15), our finding of a strong maintenance of balanced levels of substrate to catabolite in plasma from both breast cancer patients and cancer-free donors was unexpected. This may be attributable to the strong ability of the kidneys to handle increased kynurenine and it is documented that increased TDO/IDO activity does not necessarily result in increased plasma kynurenine (33, 34). Interestingly, we found that compared with healthy donors, tryptophan was only significantly lower in the plasma of patients with ER⁻ tumors. This finding suggests a particularly important role of elevated tumor tryptophan catabolism in ER⁻ disease. An expanded follow-up metabolomic study that includes more patients with ER⁻ and stage III/IV disease is imperative to determine whether plasma tryptophan and kynurenine reflect tumoral enzymatic activity and correlate with outcome. Although currently limited by lack of quality antibodies for IHC, it is likely that measurement of TDO/IDO protein levels or activity in primary tumor and/or circulating tumor cells would be more informative than plasma levels of tryptophan and kynurenine. In addition, levels of the enzymes in tumors should be correlated with tumor-infiltrating lymphocyte composition in future studies. It is possible that further flux through the kynurenine pathway in tumor cells could account for the comparatively low kynurenine concentration in plasma from women with breast cancer. In a previous study, we found that expression of *KYNU*, the gene encoding kynureninase (an enzyme downstream of TDO) is elevated in suspended TNBC cell lines and in the context of increased inflammatory signaling (10), which would be consistent with tryptophan catabolism further down the pathway. Indeed, our finding that the tryptophan/nicotinamide ratio was significantly reduced in breast cancer patient plasma compared with controls is consistent with the possibility of an overall increase in tryptophan catabolic activity not limited to the initial step in the pathway. Metabolomic tracing of tryptophan could help elucidate the ultimate fate of this amino acid in tumors.

Development of better antibodies to measure TDO and IDO or gauge activity will be a significant hurdle to overcome in the quest for biomarkers indicative of patients who may benefit from tryptophan catabolism-targeted therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L.I. Greene, T.C. Bruno, V.F. Borges, J.E. Slansky, J.K. Richer

Development of methodology: L.I. Greene, T.C. Bruno, A. D'Alessandro, J.K. Richer

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. D'Alessandro, R. Culp-Hill, J.K. Richer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.I. Greene, T.C. Bruno, J.L. Christenson, R. Culp-Hill, K. Torkko, V.F. Borges, J.K. Richer

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