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

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Abstract

Anoikis is programmed death of epithelial cells triggered by detachment from a basement membrane or extracellular matrix, and anoikis resistance is a critical step in metastasis. Triple-negative breast cancers (TNBC) have a high rate of metastasis in the first 3 years following diagnosis, and although TNBC cell lines are more resistant to anoikis than estrogen receptor positive lines, little is known regarding pathways that support anoikis resistance. Gene expression and metabolomic profiling of TNBC cells in forced suspension culture revealed multiple genes in the kynurenine pathway of tryptophan catabolism upregulated by TNBC cells in suspension, including tryptophan 2,3-dioxygenase (TDO2). Increased production of kynurenine, a key metabolite of this pathway, by TNBC in suspension activated aryl hydrocarbon receptor (AhR) transcriptional activity. Pharmacological inhibition or knockdown of TDO2 decreased kynurenine production, increased anoikis sensitivity, and inhibited proliferation, migration, and invasion. Likewise, AhR inhibition or knockdown also decreased proliferation, migration, and anchorage-independent growth. Mining publically available data, *TDO2* was found to be higher with increasing grade, higher in estrogen receptor negative than positive breast cancer, and associated with shorter overall survival. This study reveals a TDO2-AhR signaling axis activated by TNBC cells in suspension in an NF-κB dependent manner, and suggests TDO2 inhibition as a targeted therapy for TNBC. Indeed, pharmacological inhibition of TDO2 activity decreased lung colonization in a preclinical model of TNBC.

Keywords

TDO2; Aryl Hydrocarbon Receptor; triple negative breast cancer; metastasis; anoikis

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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 pro-survival pathways (as reviewed in [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, has 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 kynurenine (Kyn) [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) [17–19]. IDO1 is expressed in tissues throughout the body, while 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 [21]), but in glioma, Kyn, acting in both a paracrine and 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) [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 NFκB, which promotes anoikis resistance, as well as migratory and invasive capacity. Indeed, we found that pharmacological 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.

Results

Multiple enzymes of the kynurenine pathway are up-regulated 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 to 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 (Supplemental Figure 1A). Ingenuity Pathway Analysis of these data revealed two related pathways strongly upregulated in suspension: tryptophan catabolism and AhR signaling (Supplemental Figure 1B and 1C).

Figure 1A shows unsupervised hierarchical clustering of the top 35 genes significantly upregulated by at least 2-fold in suspended compared to 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 (Figure 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 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). *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* (Supplemental Figure 3B).

Aryl Hydrocarbon Receptor expression and activity is 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 (Figure 1A and Supplemental Figure 4). Increased *AhR* mRNA expression in suspended compared to attached TNBC cells was confirmed by qRT-PCR. Similar to *TDO2* and *KYNU*, *AhR* was not significantly increased in ER+ cell lines in suspension (Figure 2A). Furthermore, AhR protein was increased in all three TNBC cell lines tested following 24hrs in suspension as tested by western blot (Figure 1D and Supplemental Figure 3A), and in BT549 cells grown in suspension for 48hrs as measured by IHC (Figure 2C). In attached SUM159 cells, while AhR protein was found in both the cytoplasmic and nuclear fractions, after 48hrs in forced suspension AhR protein was almost exclusively nuclear (Figure 2B). Since 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 (DREs), the consensus AhR binding site. In BT549 and MDA-231 cells, luciferase activity was upregulated more than two-fold in suspended compared to attached cells (Figure 2D). The AhR antagonis α-naphthoflavone inhibited the suspension-mediated

increase in luciferase activity, demonstrating specificity of the reporter construct for AhR activity (Figure 2D and Supplemental Figure 3C).

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 10uM 680C91, which prevented the suspension-induced increase in Kyn, significantly decreased AhR activity in BT549 and MDA-MB-231 cells in suspension (Figure 2E and Supplemental Figure 3D). 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 (Figure 2F), linking TDO2 activity to AhR transcriptional activity. Addition of 100μM exogenous Kyn also resulted in a significant increase in AhR luciferase reporter activity in TNBC cells in both attached and suspended culture conditions (Figure 2G), demonstrating that Kyn is able to activate AhR in TNBC cells.

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

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.

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 (Figure 4A). MDA-MB-231, BT549, and SUM159 cells were also treated with a range of 680C91 concentrations, and GI50 values were calculated as 20 μM, 61 μM, and 102 μM, respectively (Supplemental Figure 5). Knockdown of TDO2 or AhR similarly resulted in decreased

growth of MDA-231 cells (Supplemental Figure 6), demonstrating a role for TDO2 and AhR signaling in baseline growth of TNBC cells *in vitro* even in attached culture.

Additionally, 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). 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).

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 10μM of the TDO2 inhibitor 680C91 or the AhR inhibitor CH-223191. After 24hrs, 25,000 viable cells were plated in a Matrigel-coated trans-well invasion chamber with continuous treatment. Cells grown in suspension for 24hrs were significantly more invasive than control cells grown in the attached condition (Figure 4E). The addition of the TDO2 inhibitor 680C91 during suspension culture greatly decreased the invasive capacity of viable cells. While the cells treated with the AhR inhibitor showed decreased invasion, this effect was not statistically significant (Figure 4E).

NFκB regulates expression of kynurenine pathway genes and *AhR* in TNBC cells

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

To investigate the link between increased NFkB activity, *TDO2*, and *KYNU*, we treated BT549 cells with a cocktail of TNFα and IL-1β to activate NFκB and found that expression of *TDO2*, *KYNU*, and *AhR* (Figure 5B), as well as the NFκB target genes *IL-6* and *IL-8* (data not shown) were significantly increased at 24 hrs. Next we tested whether inhibition of NFκB in suspension could reduce upregulation of Kyn pathway genes and *AhR* in TNBC cells grown in suspension. Upstream inhibition of NFκB with the Iκκ inhibitor PS1145 significantly reduced the suspension-induced increase in *TDO2, KYNU*, and *AhR* (Figure 5C), as well as well as the NFκB target gene *IL-6* (not shown), demonstrating that NFκB activity mediates the increased *TDO2, KYNU*, and *AhR* expression in TNBC cells in suspension. Lastly, a constitutively active form of IκB, a negative regulator of NFκB, was expressed in BT549 cells and this ablated the suspension-induced increase in *TDO2, KYNU,* and *AhR* (Figure 5D). These data demonstrate that the increased transcription of *TDO2, KYNU*, and *AhR* in suspension is specifically induced by and dependent on NFkB 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 48hrs in the presence of either vehicle control or the TDO2 inhibitor 680C91. 250,000 viable cells, as determined by trypan blue staining, were then injected into the tail vein of NOD/SCID mice and luminescence was monitored over time. Seven days after injection, mice that received vehicle-treated cells had significantly higher luminescence, and this statistically significant difference was maintained throughout the experiment (Figure 6A–B). At the conclusion of the experiment at Day 28 post-injection, lung luminescence was measured *ex-vivo*, and lungs from mice that received vehicle-treated cells had significantly higher luminescence compared to mice that received cells treated with the TDO2 inhibitor (Figure 6C). A significant decrease in the number of metastatic nodules in the lungs from mice receiving cells treated with 680C91 was also observed by H&E (Figure 6D). Together, these data demonstrate that TDO2 inhibition decreases the ability of TNBC cells to successfully metastasize following tail vein injection *in vivo*.

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 Figure 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 *et al* dataset [37] had the largest patient population (n=1998) and was therefore selected for analysis. Notably, *TDO2* was the 6th most highly-overexpressed gene in breast carcinoma compared to normal breast tissue (top 1% of overexpressed genes, p<0.0001; Figure 7B). *TDO2* gene expression was significantly higher in ER-negative breast tumors compared to ER-positive tumors (p<0.0001; Figure 7C). High *TDO2* expression was also associated with increasing grade (p<0.001; Figure 7D), supporting the hypothesis that tumors with high *TDO2* expression may have increased metastatic potential. Similarly, *IDO1* was overexpressed in breast carcinoma compared to normal breast tissue, although it was not as highly overexpressed as *TDO2* (Supplemental Figure 7A). *IDO1* was also more highly expressed in ER− than ER+ breast tumors in both the Curtis and TCGA data sets (Supplemental Figure 7B–C).

Finally, patients whose tumors had above-median *TDO2* expression had approximately 3 years shorter overall survival (OS) when compared to those with below-median *TDO2* expression (median OS 10.62 years vs. 13.31 years, respectively; p=0.0002, log-rank test; Figure 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 (Supplemental Figure 7D). 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 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 (Figure 7F). Central to this model is upregulation of TDO2, a key enzyme in the Kyn pathway. Our observation that pharmacological inhibition of TDO2 decreased the ability of MDA-MB-231 cells to form lung 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 NFkB in suspension. We previously identified a different NFκB-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 NFκB signaling in suspended cells supports multiple pro-metastatic 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 underway, 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 anti-tumor immune response. However, 1-MT does not target TDO2 [40]. Importantly, our data combined with data mined from publically 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]. While this finding was attributed to IDO1 activity, we find that that *TDO2* is higher in ER− tumors than ER+ in two publically available data sets, 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 (Figure 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 publically available data sets correlates with shorter overall survival. *TDO2* was significantly upregulated in metastases of leiomyosarcoma patients compared to primary tumors [43], and increased *KYNU* expression is associated with metastasis of breast cancer cells to the lung and brain [44, 45]. In addition to abrogating the autocrine pro-tumorigenic 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.

While 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 is 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 suggests 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 recognized 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 pro-tumorigenic autocrine effects and potential immunosuppressive paracrine signaling effects make 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 pharmacological 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.

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% fetal bovine serum, 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% fetal bovine serum, 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% fetal bovine serum, penicillin/streptomycin and insulin. MCF7 and T47D cells were purchased from the ATCC, and were grown in DMEM with 10% FBS, and 2 mM 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 HPLC

Experimental samples were generated by plating 500,000 cells in 2mL culture media in a 6-well plate in triplicate. After 48hrs, media was 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 HPLC with a Kinetex BiPhenyl column (particle size 2.7 μm, 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 to 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, 100uL 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, Bristol, United Kingdom) 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. IL-1β and TNF-α were obtained from eBioscience (San Diego, CA).

Cellular Fractionation

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

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Plus kit (QIAGEN) according to manufacturer 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 biological triplicate. For primer sequences, see Supplemental Methods.

Immunoblotting

Whole cell protein extracts (50 μg) were denatured, separated on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. After blocking in 3% bovine serum albumin in Tris-buffered saline–Tween, membranes were probed overnight at 4°C. Primary antibodies used include: AhR (#13790, 1:1000 dilution; Cell Signaling Technology), KYNU (H00008942-B01, 1:1000 dilution; NOVUS Biologicals, Littleton, CO), TDO2 (H00006999-B01P, 1:1000 dilution; Abnova, Taipei, Taiwan), TOPO1 (C-21, 1:100 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX), 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; Waltham, MA). Densitometry quantification was performed using Image Studio Lite Version 3.1 and reported as a ratio compared to α-Tubulin as a loading control.

Immunohistochemistry

5μm FFPE sections were heat immobilized onto glass slides and deparaffinized in a series of xylenes and graded ethanols. Antigens were heat retrieved in 10mM Tris, 1mM 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 RT at 1:200 in TBST, and AhR antibody (CST #13790) was incubated for 1h at RT at 1:50 in TBST. Envision polymer (Dako, Carpinteria, CA) 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 hrs. 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 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 versus suspended). Fold-change cut-off was 1.5 and significance cut-off was p<0.05. Significantly differentially expressed genes were imported to Ingenuity for pathway analysis, including identification of altered canonical pathways.

Short Hairpin RNA (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. 24 hours after plating, 100 ul of the lentiviral suspension and 8 ug/ml polybrene (Sigma-Aldrich) was added to the tissue culture media. After 24 hours, viral media was replaced with regular tissue culture media and cells were incubated for an additional 24 hours before the commencement of puromycin selection (1 ug/ml). Knockdowns were confirmed by western blot and all experiments were done within three passages of puromycin selection. For shRNA sequences, see Supplemental Methods.

Luciferase Reporter Activity

The AhR luciferase reporter, generously provided by Dr. Michael Denison (University of California-Davis), and NFκB 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 24hr. 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 15min at room temperature using 1X passive lysis buffer. Lysed cells were collected and centrifuged at 14,000rpm for 15 minutes at 4°C to eliminate cell debris. The supernatant was used immediately or diluted with 1X passive lysis buffer for determination of luciferase activity. For analysis, AhR or NFκB 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 was refreshed every 4 days.

Caspase-3/7 activity was measured using Caspase-Glo® 3/7 Assay kit (Promega, Madison, WI, USA) according to 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 RT for 30 min. Luminescence was determined by luminometer, measuring luminescence after 1sec.

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 48hrs in the presence of either vehicle control or 10 μM 680C91. 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, Bar Harbor, ME). 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, Ann Arbor, MI). 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; Bedford MA) per the manufacturer's protocol.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5. Student's 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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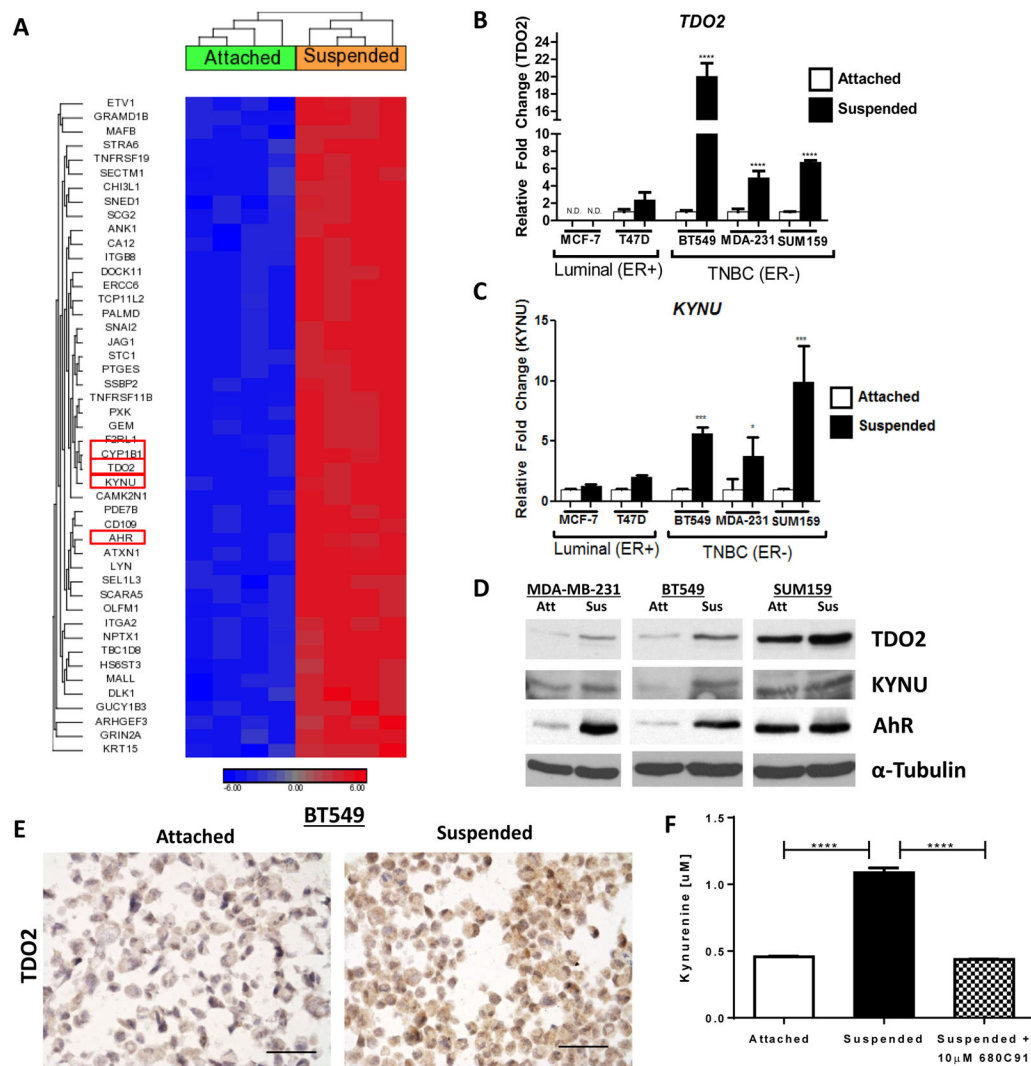
Figure 1. Kynurenine pathway components and activity are increased in TNBC cells in suspension and suppressed by TDO2 inhibition

(**A**) BT549 cells were grown in either attached or forced-suspension conditions on poly-HEMA coated plates in biological quadruplicate for 24h. RNA was harvested at 24h and hybridized onto Affymetrix Human Gene 1.0ST array. Gene expression profile analysis obtained by analysis identified genes significantly upregulated in suspension culture (p<0.05). Those involved in tryptophan metabolism and AhR signaling highlighted in red. (**B**) Relative TDO2 and (**C**) KYNU mRNA levels determined by qRT-PCR in luminal and TNBC cell lines grown in attached or suspended culture for 24h. (**D**) Western blot for TDO2, KYNU, and AhR protein in three TNBC cell lines following 24h of attached or suspended culture. (**E**) TDO2 protein detected by immunohistochemistry in BT549 cell pellets grown in attached or suspended culture for 48h. Bar equals 50μm. (**F**) Secreted kynurenine levels, as measured by HPLC, from BT549 cells grown for 48h in the attached versus suspended conditions or suspended plus 10μM 680C91 (a specific TDO2 inhibitor). *P<0.05, ***P<0.001, ****P< 0.0001 by ANOVA with Bonferroni's Multiple Comparison Test.
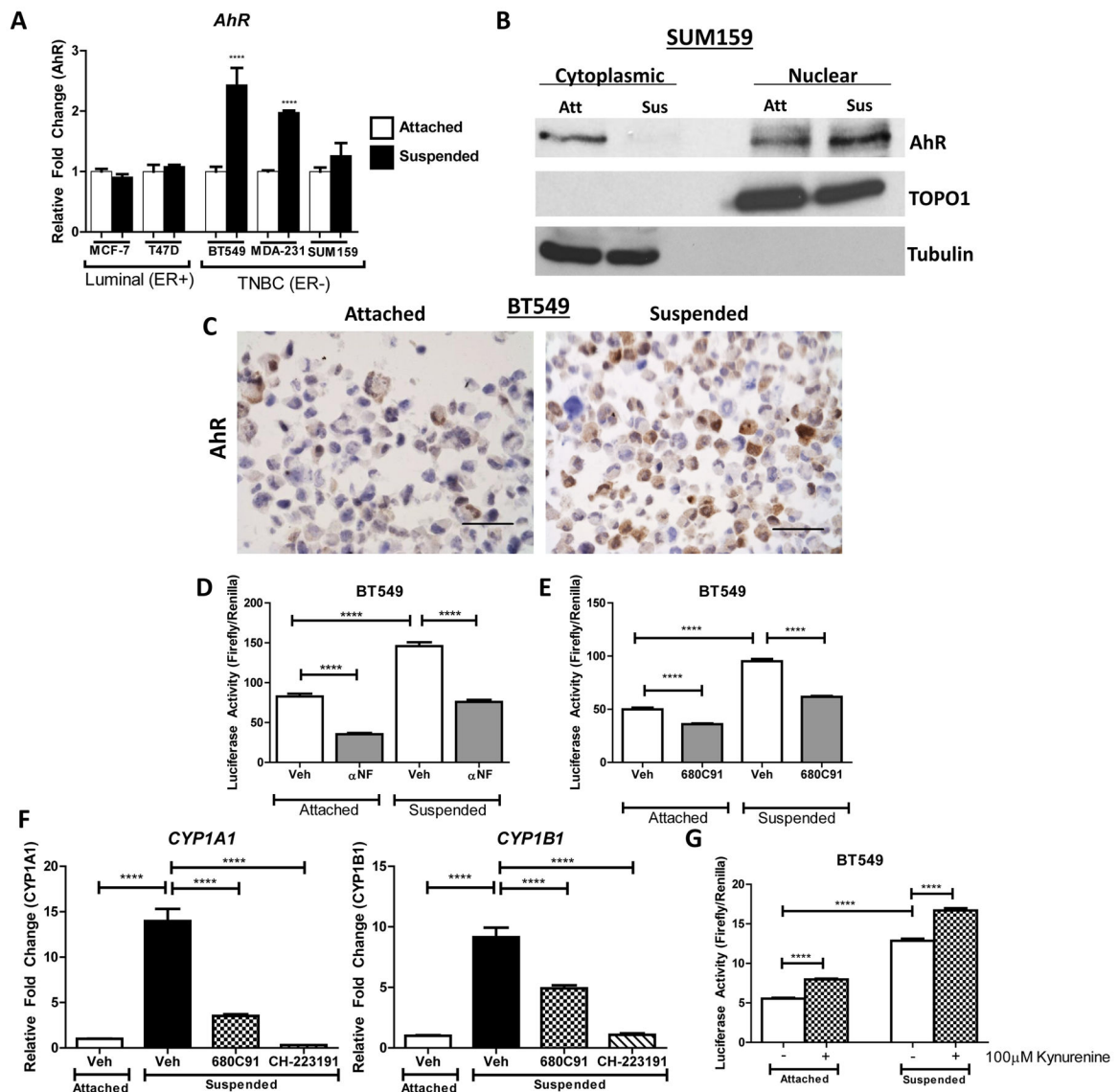
Figure 2. AhR expression and localization is affected by suspension culture, and kynurenine activates AhR in TNBC cells in suspension

Breast cancer cell lines were plated on uncoated (attached) or polyHEMA-coated (suspended) tissue culture plates for 24 h. **(A)** Relative *AhR* levels measured by qRT-PCR in TNBC cells in attached or suspended culture for 24h. **(B)** AhR levels in nuclear and cytoplasmic fractions of SUM-159 cells in attached or suspended culture for 24h. **(C)** AhR protein as detected by immunohistochemistry in BT549 cell pellets grown in attached or suspended culture for 48h. Bar equals 50μm. **(D and E)** AhR reporter activity in BT549 cells in attached or suspended culture for 24h treated with **(D)** vehicle (0.1% DMSO) or 10μM α-Naphthoflavone (AhR antagonist) or **(E)** vehicle (0.1% DMSO) or 10μM 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

24h treated with vehicle (0.1% DMSO) or crystalline kynurenine (100uM). *p<.05, **p<.01, ****p < .0001 by ANOVA with Bonferroni's Multiple Comparison Test.
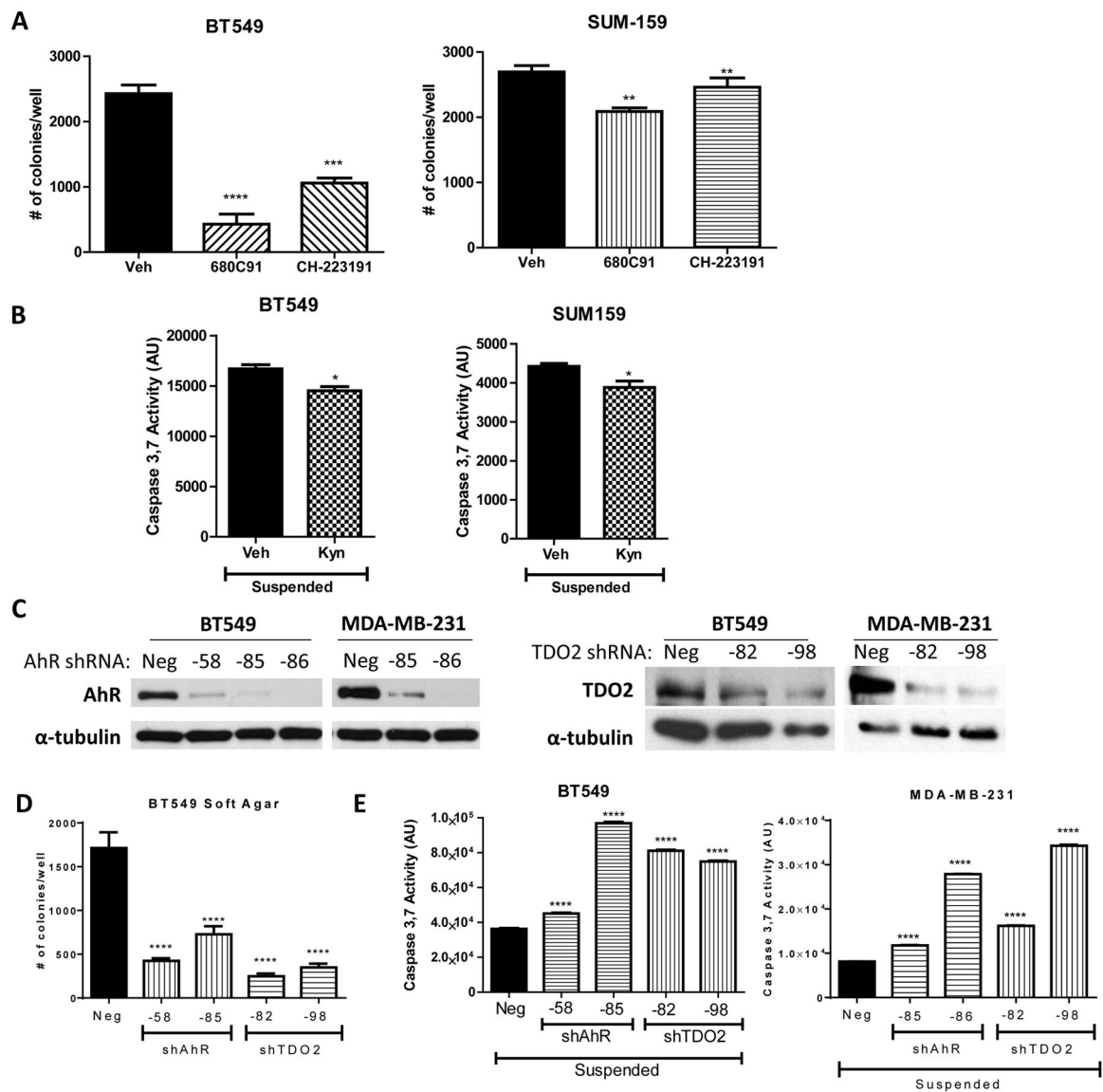
Figure 3. Targeting the kynurenine pathway or AhR increases cell death of TNBC cells in suspension

(A) Anchorage-independent growth of SUM-159 or BT549 cells pre-treated with vehicle (0.01% DMSO), 10μM 680C91 (TDOi), or 10μM CH-223191 (AhR antagonist) for 24h, then plated in 0.25% soft agar where respective treatments were maintained for 18–21 days. **(B)** Caspase 3/7 activity in TNBC cells (BT549 and SUM159) treated with either vehicle control or 100μM Kynurenine while cultured in suspension for 48h. **(C)** Western Blot of TDO2 or AhR in attached BT549 cells following transduction with non-targeting 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 cells transduced with non-targeting shRNA (shNEG) or shRNA constructs targeting AhR or TDO2, and plated in poly-HEMA coated

plates for 48h. **P<0.01, ****P< 0.0001 by ANOVA with Bonferroni's Multiple Comparison Test.
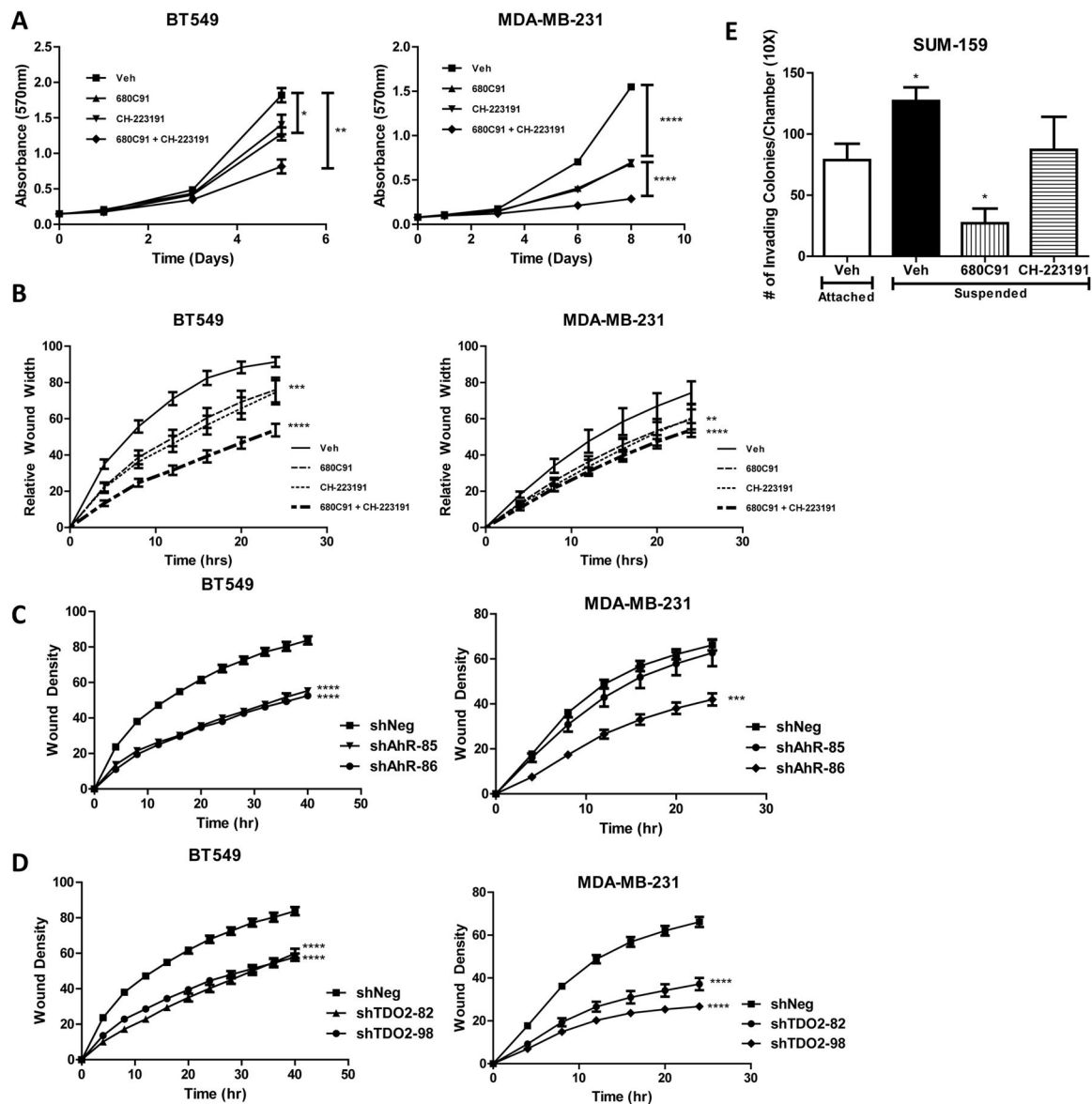
Figure 4. TDO2 and AhR inhibition decrease cell number, migration, and invasion of TNBC cells

BT549 and MDA-MB-231 cells (TNBC) were treated with either vehicle control (0.01% DMSO), 10μM 680C91 (TDOi), 10μM 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–D)** Scratch wound assay of BT549 and MDA-MB-231 cells transduced with a non-targeting control (shNeg) or shRNAs targeting **(C)** AhR (shAhR-85, shAhR-86) or **(D)** TDO2 (shTDO2-82, shTDO2-98). **(E)** Boyden chamber invasion assay of SUM-159 cells pretreated in the attached or suspended conditions for 24h then seeded into the upper chamber and allowed to invade through a matrigel-coated membrane. *p<.05, **p<.01, ****p < .0001 by ANOVA with Bonferroni's Multiple Comparison Test.
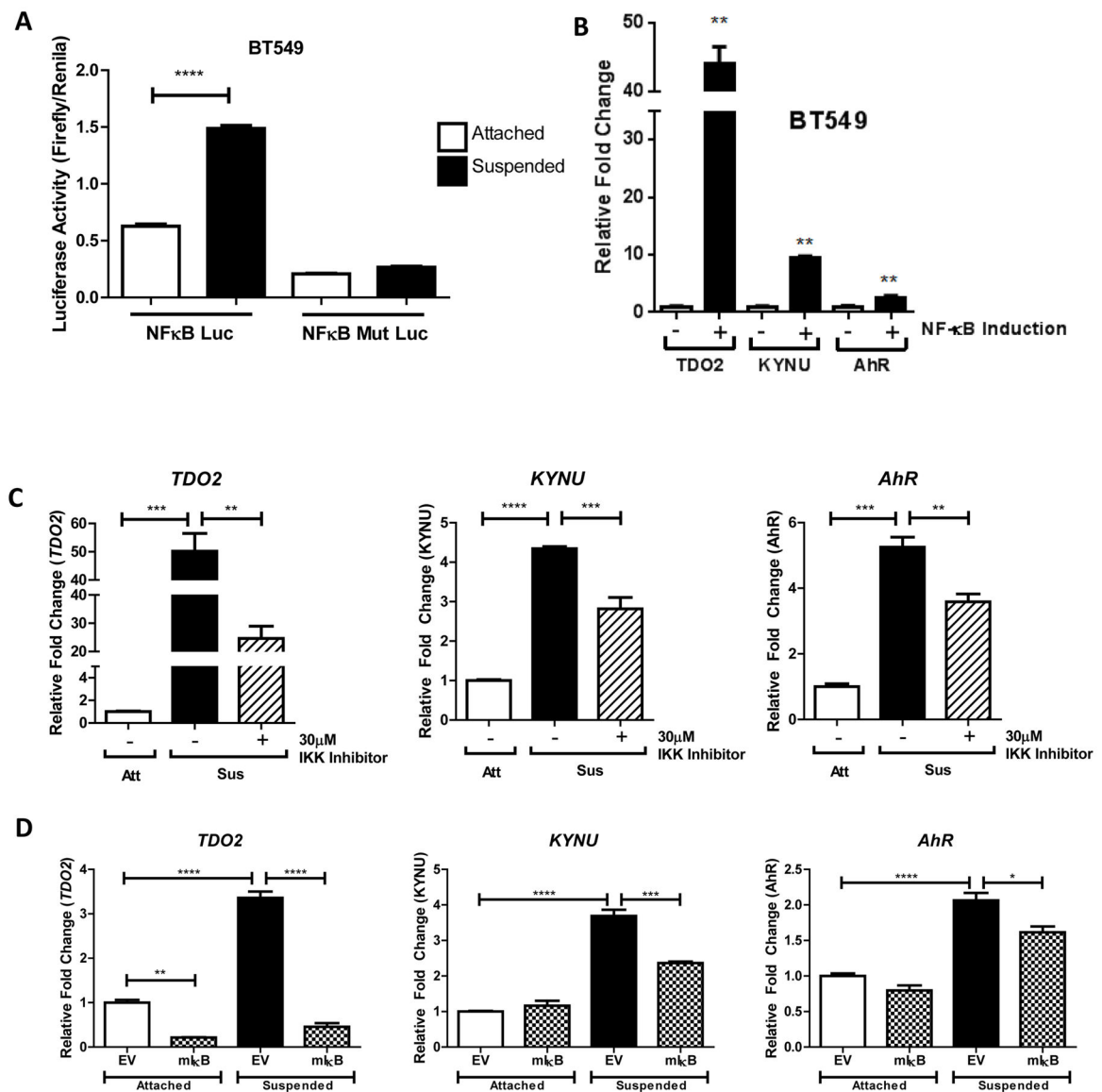
Figure 5. NFκB regulates expression of TDO2, KYNU, and AhR in TNBC cells in suspension
(**A**) Activity of an NFκB (left) or mutated NFκB (right) luciferase reporter in BT549 cells in attached or suspended condition for 24h. (**B**) Relative expression of *TDO2, KYNU,* and *AhR* by qRT-PCR in BT549 cells treated with vehicle or TNFα (10ng/ml) plus IL-1β (10ng/ml) for 24h. (**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 30μM PS1145 (IKK inhibitor) for 24h. (**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's Multiple Comparison Test.
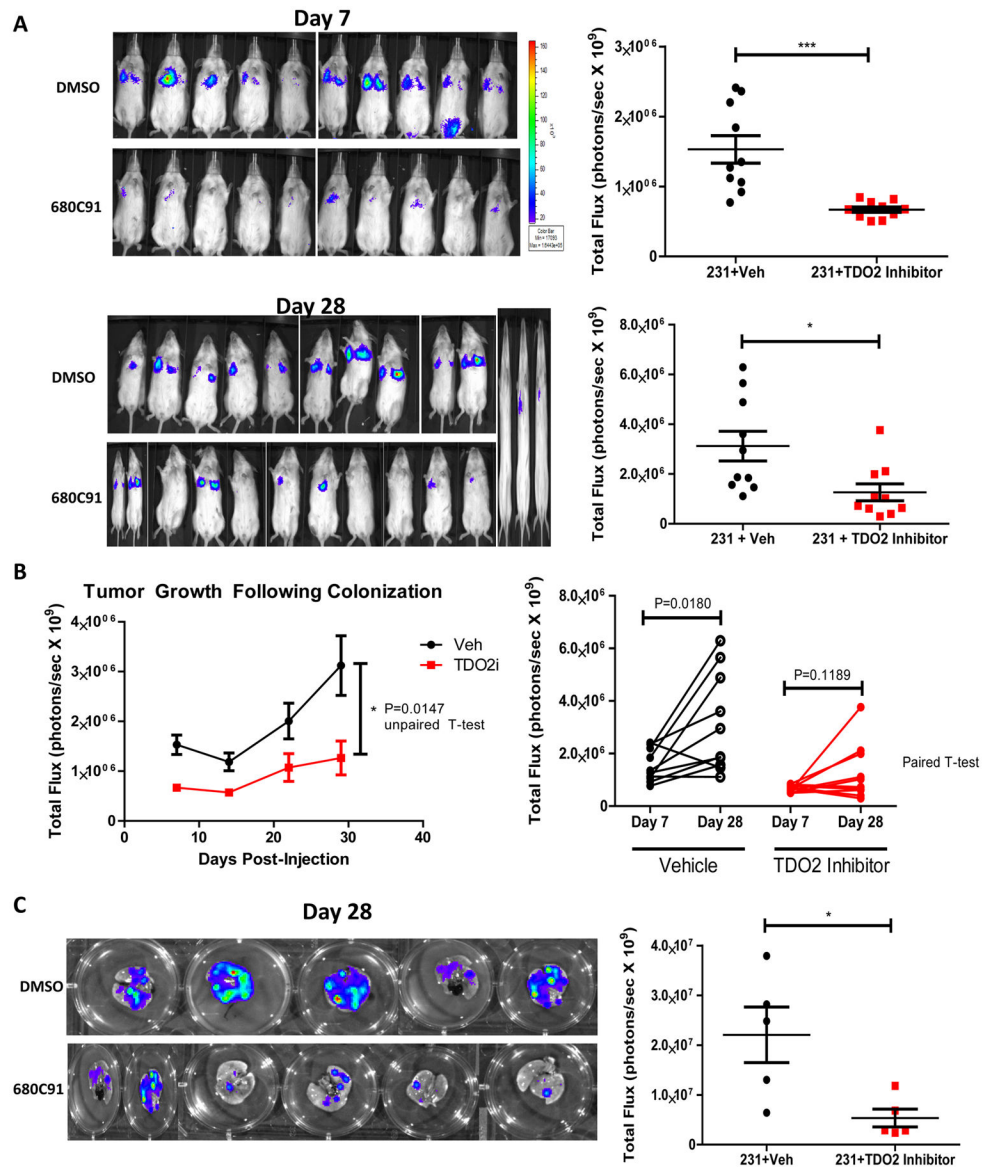
Figure 6. Targeting TDO2 decreases colonization and outgrowth of TNBC cells *in vivo*
Luciferase-expressing MDA-MB-231 cells were pretreated for 48 hours in forced-suspension culture with either vehicle (0.01% DMSO) or 10μM 680C91 prior to tail vein injection. **(A–B)** Metastatic colonization was measured by whole animal IVIS imaging at 7 days post-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.
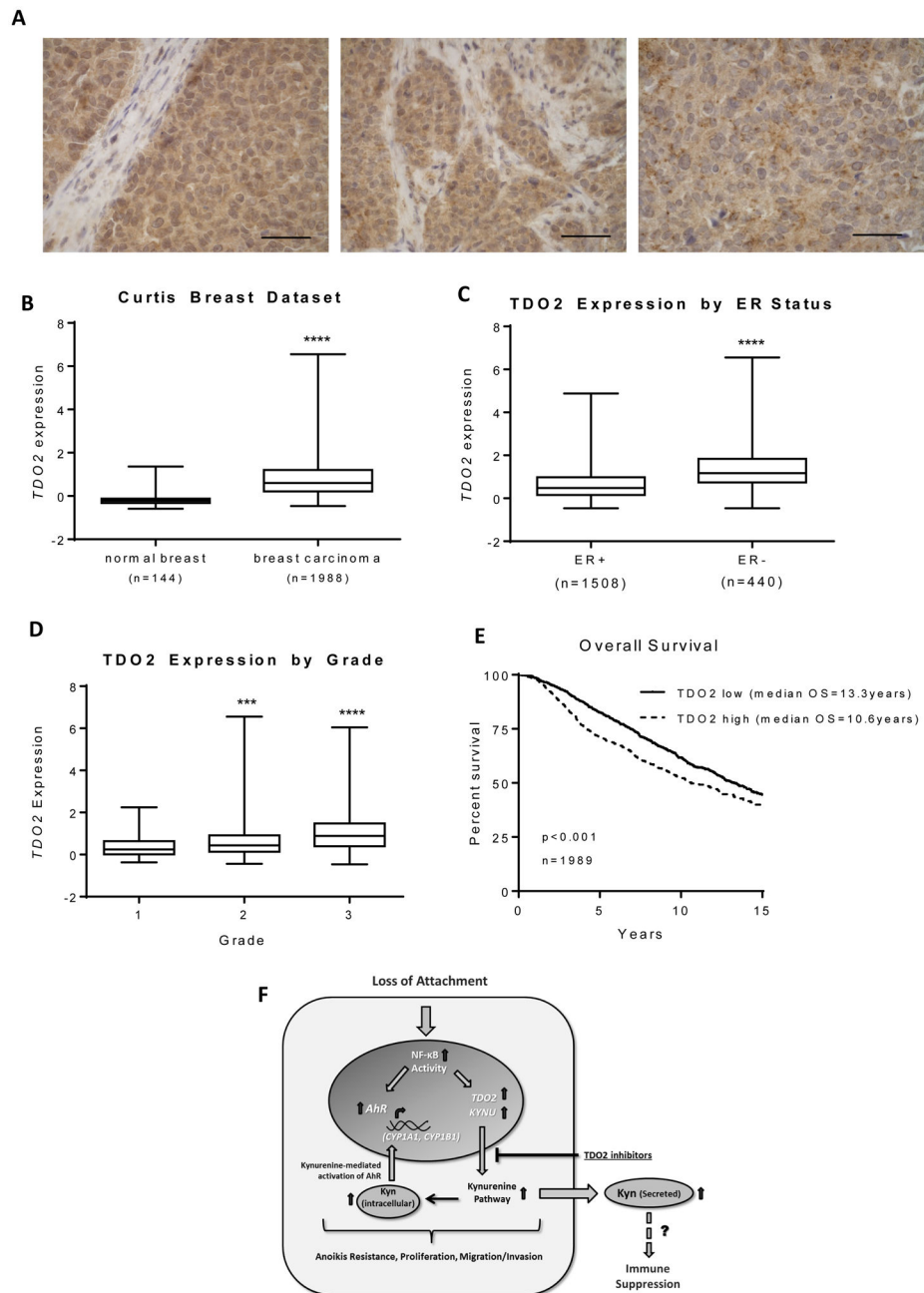
Figure 7. *TDO2* expression in breast cancer clinical samples
(A) TDO2 staining in primary TNBC patient samples, bar equals 50 μm. **(B–E)** Using the Curtis Breast Cohort, *TDO2* expression was compared between normal breast and invasive ductal carcinoma **(B)**. *TDO2* gene expression in breast tumor samples divided by **(C)** ER+ versus ER−, or **(D)** by tumor grade. **(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: NFκB 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<.001 by log-rank test.