



ORIGINAL ARTICLE

Stemness and immune evasion conferred by the TDO2-AHR pathway are associated with liver metastasis of colon cancer

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Abstract

The aryl hydrocarbon receptor (AHR) pathway modulates the immune system in response to kynurenine, an endogenous tryptophan metabolite. IDO1 and TDO2 catalyze kynurenine production, which promotes cancer progression by compromising host immunosurveillance. However, it is unclear whether the AHR activation regulates the malignant traits of cancer such as metastatic capability or cancer stemness. Here, we carried out systematic analyses of metabolites in patient-derived colorectal cancer spheroids and identified high levels of kynurenine and *TDO2* that were positively associated with liver metastasis. In a mouse colon cancer model, *TDO2* expression substantially enhanced liver metastasis, induced AHR-mediated *PD-L1* transactivation, and dampened immune responses; these changes were all abolished by *PD-L1* knockout. In patient-derived cancer spheroids, *TDO2* or AHR activity was required for not only the expression of *PD-L1*, but also for cancer stem cell (CSC)-related characteristics and Wnt signaling. *TDO2* was coexpressed with both *PD-L1* and nuclear β -catenin in colon xenograft tumors, and the coexpression of *TDO2* and *PD-L1* was observed in clinical colon cancer specimens. Thus, our data indicate that the activation of the TDO2-kynurenine-AHR pathway facilitates liver metastasis of colon cancer via *PD-L1*-mediated immune evasion and maintenance of stemness.

KEYWORDS

aryl hydrocarbon receptor, cancer stem cells, kynurenine, liver metastasis, TDO2

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1 | INTRODUCTION

Colorectal cancer is the third leading cause of cancer-related death worldwide (<https://gco.iarc.fr/today/fact-sheets-cancers>). The presence of distant metastatic lesions leads to a dismal prognosis with the 5-year survival rate at 14% (<https://www.cancer.net/cancer-types/colorectal-cancer/statistics>) due to chemoresistance to standard chemotherapy and unfavorable responses to immunotherapy.¹ Among the types of distant metastasis in colorectal cancer, liver metastasis is the most frequently observed type and directly linked to the high mortality rate. Hence, understanding how liver metastasis develops is a pressing medical issue.

It was reported that liver metastasis was effectively blocked by the elimination of colon cancer cells that express *LGR5*, an established functional marker of colon cancer stem cells (CSCs),^{2,3} suggesting that cancer cells with enhanced stemness play an important role in the liver metastasis of colon cancer. Effective inhibition of liver metastasis by the elimination of *LGR5*-positive cells³ indicate that targeting *LGR5*-positive CSCs may be a promising strategy to effectively treat liver metastasis.

In our previous studies, we established spheroid cultures from clinical specimens of primary colorectal cancer.⁴ The established spheroids were mainly composed of cells with CSC-related characteristics including self-propagating capability and expression of CSC-related markers (eg, CD44 and activated β -catenin) and capable of forming xenograft tumors with pathological features similar to those of the original cancers.⁵

Here, to gain insight into the biological features of colorectal CSCs that are associated with metastatic capability, we attempted to identify cellular metabolites that were upregulated in spheroids derived from primary tumors of patients with liver metastasis as well as from metastatic lesions of the liver. One of metabolites significantly upregulated in the metastasis-associated spheroids was kynurenine, a major tryptophan metabolite produced mainly by its catalyzing enzymes, indoleamine-2,3-dioxygenase 1 (IDO1) and tryptophan-2,3-dioxygenase (TDO2).

It was demonstrated that elevation of kynurenine suppresses antitumor immunity through activation of aryl hydrocarbon receptor (AHR).⁶ AHR is a ligand-dependent transcription factor that responds to various exogenous and endogenous compounds.^{7,8} Upon binding to these diverse forms of ligands, activated AHR can modulate immune functions, thus playing a major role in fine-tuning the immune system.^{7,8}

Because of documented roles of kynurenine in cancer progression, we further examined the significance of upregulation of kynurenine in metastatic spheroids. Our study revealed the functional importance of the kynurenine-regulated TDO2-AHR pathways in maintenance of stemness and PD-L1-mediated immunomodulation, which may translate into generation of immune-evasive CSCs with metastatic capability.

2 | MATERIALS AND METHODS

2.1 | Primary human colon cancer specimens

All human colon cancer samples were resected from patients who provided informed consent at the National Cancer Center Hospital, and all procedures were conducted under a protocol approved by the ethics committee of the National Cancer Center. Clinical information of each case for the examined spheroids were provided in Figure S1A.

2.2 | Animal experiments

All mouse procedures were approved by the Animal Care and Use Committees of the National Cancer Center and conducted in accordance with institutional policies and the Guidelines for Animal Experiments.

3 | RESULTS

3.1 | Elevated kynurenine and TDO2 levels in patient-derived colorectal cancer spheroids are associated with liver metastasis

We established patient-derived spheroids from metastatic liver lesions by using previously described protocol.⁴ To identify metabolites with elevated expression levels in metastatic cancer, we cultivated one set of spheroids from the primary tumors of patients without metastasis (CRC-9, 17, 19, and 20, hereafter called nonmetastatic spheroids) and another set of the newly established spheroids from liver metastases (CRC-29 M, 31 M, 24 M, 26 M, hereafter called metastatic spheroids) (Figure S1A). After extracting metabolites from the lysates of those spheroids, we compared the levels of metabolites by systematically quantifying the charged metabolites by capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) (Figure 1A).⁹ The 88 cationic metabolites (Table S1) and 126 anionic metabolites (Table S2) that were detected in any of the sample lysates were then compared; we found that the levels of two metabolites in the metastatic spheroids, ie, kynurenine (a cationic metabolite) and octanoate (an anionic metabolite), were significantly higher in lysates in the metastatic spheroids than those in the nonmetastatic ones (Figures 1B and S1B; Tables S3 and S4).

We then confirmed the elevation in kynurenine levels in the metastatic spheroids by performing mass spectrometry with a different separation technique, gas chromatography tandem mass spectrometry (GC-MS/MS). This technique was used because it does not require nonisotonic washing, and we found significantly reduced spheroid viability in CE-TOF-MS (see Supplemental Methods section). We measured kynurenine levels by GC-MS/MS in samples

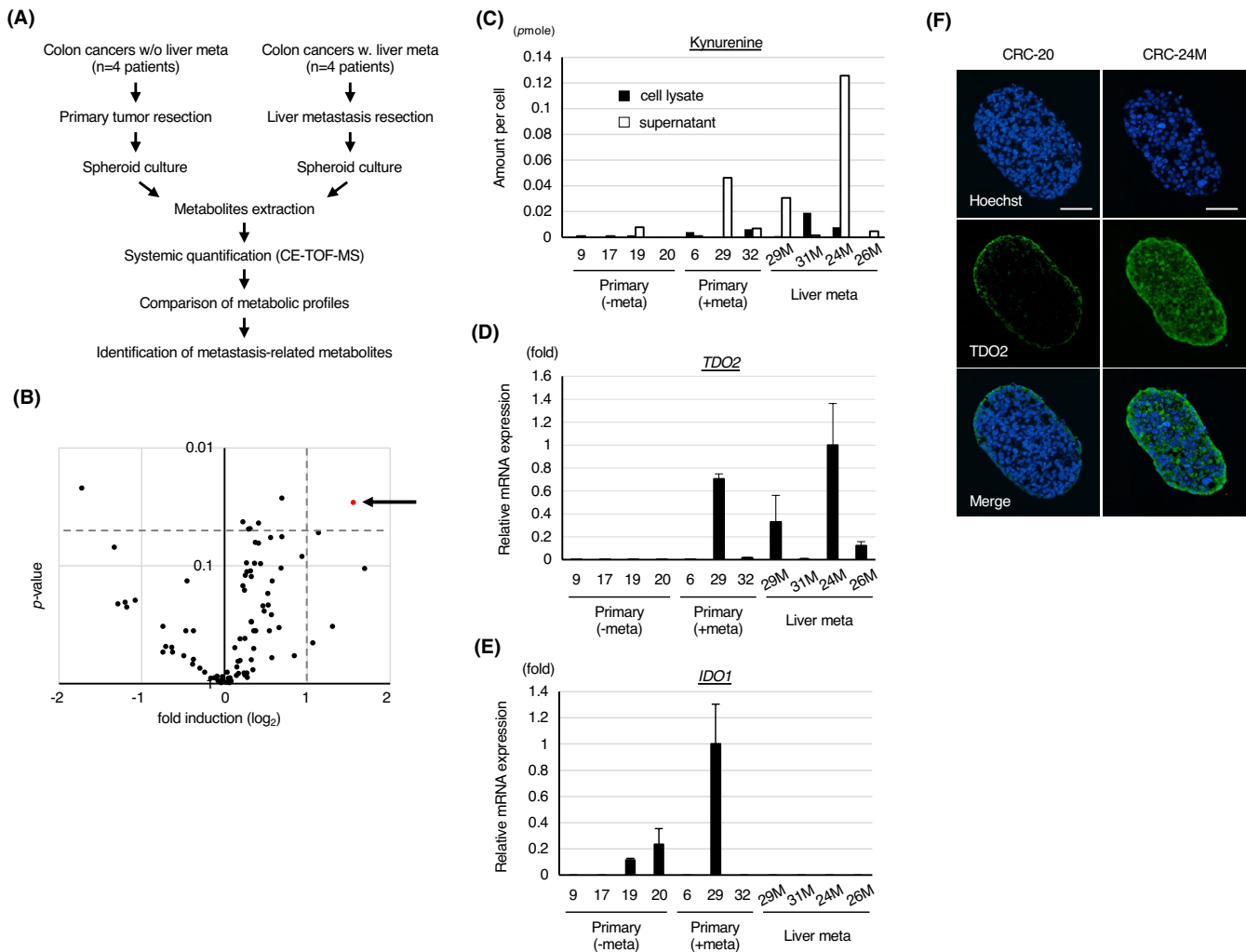


FIGURE 1 Elevated TDO2-kynurenine pathway activity in patient-derived colon cancer spheroids is associated with liver metastasis. A, Experimental scheme. B, Volcano plot of the quantified concentration for cationic metabolites through the comparison of nonmetastatic spheroids vs. metastatic spheroids. L-kynurenine is indicated by the arrow. C, Levels of kynurenine per cell measured by chromatography tandem mass spectrometry (GC-MS/MS). D, E, qPCR analyses of (D) *TDO2* and (E) *IDO1* in the indicated spheroids. F, Representative immunostaining for TDO2 in spheroids with a low (CRC-20) or high (CRC-24 M) level of TDO2. Nuclei were counterstained by Hoechst 33342. Scale bar: 50 μm. Values represent the mean ± SD

from spheroids derived from the primary tumors of patients with liver metastasis (CRC-6, 29, 32) (Figure S1A) in addition to samples from nonmetastatic and metastatic spheroids. Kynurenine levels were evaluated in both culture supernatant and cell lysate extracts, as kynurenine can be secreted from cells.¹⁰ The data obtained by GC-MS/MS confirmed that the kynurenine levels tended to be higher in the metastasis-associated spheroids than in the nonmetastatic spheroids (Figure 1C). Remarkably, we found that kynurenine levels were generally higher in the supernatants than in the cell lysates, suggesting that a majority of the produced kynurenine was secreted from the cells (Figure 1C).

Kynurenine is produced mainly by two catalytic enzymes, IDO1 and TDO2 (Figure S1C).⁶ The expression of *TDO2* but not *IDO1* was upregulated in metastasis-associated spheroids with high levels of kynurenine production, suggesting that induction of *TDO2* is responsible for the elevated kynurenine levels in the metastatic

spheroids (Figures 1D, E and S1D). Consistently, immunostaining studies showed that TDO2 protein was expressed at higher levels in metastatic spheroids (CRC-24 M) than in nonmetastatic spheroids (CRC-20) (Figure 1F).

We investigated the mutation status of 114 major cancer-associated genes by using an in-house gene panel test¹¹ (Figure S1E). We did not observe a clear association between high levels of kynurenine and mutations in major genes involved in colorectal cancer (ie, *APC*, *KRAS*, and *TP53*) nor genes associated with high-frequency microsatellite instability (MSI-H) (*MLH1*, *MSH2*, and *POLE*). Clustering analyses based on RNA-seq data indicated that the expression profiles of the spheroids were more similar to colon cancer cells in the CMS-2 and CMS-3 category than those in the MSI-H-related CMS-1 (Figure S1F), suggesting that all the examined spheroids were associated with microsatellite-stable phenotype.

3.2 | Tdo2 expression promotes liver metastasis in an Ahr-dependent manner

Next, we evaluated whether *Tdo2* overexpression affects colon cancer progression in highly immunogenic colon cancer cells, CT26.¹² Flag-tagged mouse *Tdo2* was introduced into the CT26 cells expressing GFP (CT26/GFP) via lentiviral gene transfer (Figure S2A). *Tdo2* overexpression induced an increase in the supernatant kynurenine level that was comparable to that in supernatants of human U87 cells, which express high levels of endogenous *TDO2* and kynurenine (Figure S2B).¹³ *Tdo2* overexpression did not significantly affect *Ido1* expression (Figure S2C) and did not significantly promote cell growth (Figure S2D), migration (Figure S2E, upper panel), or cell invasion (Figure S2E, lower panel). Because the activation of AHR signaling has been shown to promote EMT phenotype,¹⁴ we also examined the expression of representative EMT markers. However, *Tdo2* overexpression did not significantly enhance their expression (Figure S2F).

Next, we generated liver metastasis by injecting *Tdo2*-expressing CT26 cells into the spleen of *Balb/c* syngenic mice (Figure 2A). In vivo imaging revealed that *Tdo2* expression significantly enhanced liver metastasis (Figure 2B). The enhancement of metastasis by *Tdo2* was abolished by a specific inhibitor of *TDO2* (680C91)¹³ (Figure 2C). In addition, suppression of Ahr with a specific inhibitor (CH-223191)¹⁵ completely blocked *Tdo2*-mediated enhancement of liver metastasis (Figure 2D). These data collectively indicate that Ahr activation by *Tdo2*-produced kynurenine is responsible for the enhancement of liver metastasis of colon cancer cells.

Remarkably, splenic coinjection of RFP-labeled CT26 cells (CT26/RFP) together with CT26/*Tdo2*/GFP cells at 1:1 cell ratio enhanced liver metastasis by the CT26/RFP cells, indicating that *Tdo2*-expressing cells affect the metastatic capability of surrounding cancer cells, presumably due to elevated levels of extracellular kynurenine secreted by CT26/*Tdo2*/GFP (Figure 2E).

3.3 | Tdo2-enhanced liver metastasis is associated with compromised immune responses

Enhanced liver metastasis by *Tdo2* overexpression was not observed if the metastasis assay was performed with nude mice (Figure S3A), suggesting that the host immune response is involved in *Tdo2*-dependent liver metastasis. Hence, we examined whether *Tdo2* overexpression affects immunoenvironment in metastasized liver. The metastatic tumors formed after splenic injection of CT26/GFP cells were infiltrated by both CD4⁺ and CD8⁺ lymphocytes (Figure 3A), presumably due to an immunogenic response induced by the retroviral envelope expressed in parental CT26 cells.¹⁶ Notably, the accumulation of both CD4⁺ and CD8⁺ lymphocytes was significantly decreased in stroma surrounding *Tdo2*-expressing metastatic tumors (Figure 3A, B). In contrast, there were few infiltrating macrophages regardless of whether *Tdo2* was expressed (Figure S3B).

Consistent with the immunostaining results, FACS analyses indicated that *Tdo2* overexpression decreased the proportions of major T lymphocyte subgroups (CD3⁺, CD4⁺, CD8⁺, Treg, and IFN- γ ⁺ CD8⁺ cells) (Figure S3C). Compromised host immune responses by *Tdo2* expression may be clinically relevant, as the presence of a large number of tumor-infiltrating T lymphocytes has been shown to be a predictor of good prognosis in multiple solid tumors¹⁷ and liver metastasis.¹⁸

3.4 | The Tdo2-kynurenine-Ahr pathway mediates the transactivation of *Pd-1* and *Tdo2* in liver metastasis-competent colon cancer cells

It was demonstrated that Ahr transactivates PD-L1 in carcinogen-treated lung epithelial cells.¹⁹ Therefore, we next examined whether the *Tdo2*-dependent enhancement of liver metastasis is mediated by Ahr-dependent *Pd-1* induction. Indeed, the expression of *Pd-1* was upregulated in *Tdo2*-expressing CT26 cells (Figure 3C, D). Treatment with an inhibitor of *Tdo2* or Ahr abolished the induction of *Pd-1* and *Il-1b*, known targets of Ahr²⁰ (Figure 3E, F). Inhibition of *Pd-1* expression by the inhibitor of *Tdo2* or Ahr was also shown by Western blot analyses (Figure S3E, F).

Interestingly, the examination of the expression of the *Tdo2* gene's 3'UTR sequence revealed that endogenous *Tdo2* expression was also induced by ectopic *Tdo2* expression and that the induction was compromised by the inhibitor of *Tdo2* or Ahr (Figure 3E, F). These results suggest that Ahr activates the *Tdo2* gene, forming a positive feedback loop. As expected, in silico analyses of genomic sequences showed that the promoter regions of the *Pd-1* and *Tdo2* genes harbor potential binding sites for Ahr (Figures 3G, H and S3G). Additionally, chromatin immunoprecipitation (ChIP) analyses revealed that *Tdo2* overexpression markedly enhanced the binding of Ahr to the promoter sequences of *Pd-1*, *Tdo2*, and *Il-1b*, and that the enhanced interactions were largely abolished by the inhibitor of *Tdo2* or Ahr (Figure 3I). Collectively, these data indicate that the activation of the kynurenine-Ahr pathway by *Tdo2* induces the transactivation of *Pd-1* and *Tdo2* in colon cancer cells.

3.5 | *Pd-1* is required for the *Tdo2*-mediated promotion of liver metastasis and suppression of immune responses

Next, we investigated the functional importance of the induction of *Pd-1* in liver metastasis. We performed CRISPR/Cas9-mediated knockout of *Pd-1* in CT26/GFP/*Tdo2* cells (Figure 4A). Knocking out *Pd-1* almost completely abolished the *Tdo2*-mediated enhancement of liver metastasis (Figure 4B, C) and restored the T cell-mediated immune response in liver metastases (Figure 4D-F), while the knockout did not significantly affect the proliferation of *TDO2*-expressing tumor cells in vitro (Figure S4). *Tdo2*-mediated liver metastasis could also be abolished by intraperitoneal injection of a neutralizing anti-PD-L1 antibody (Figure 4G, H). Thus, *Pd-1* transactivation

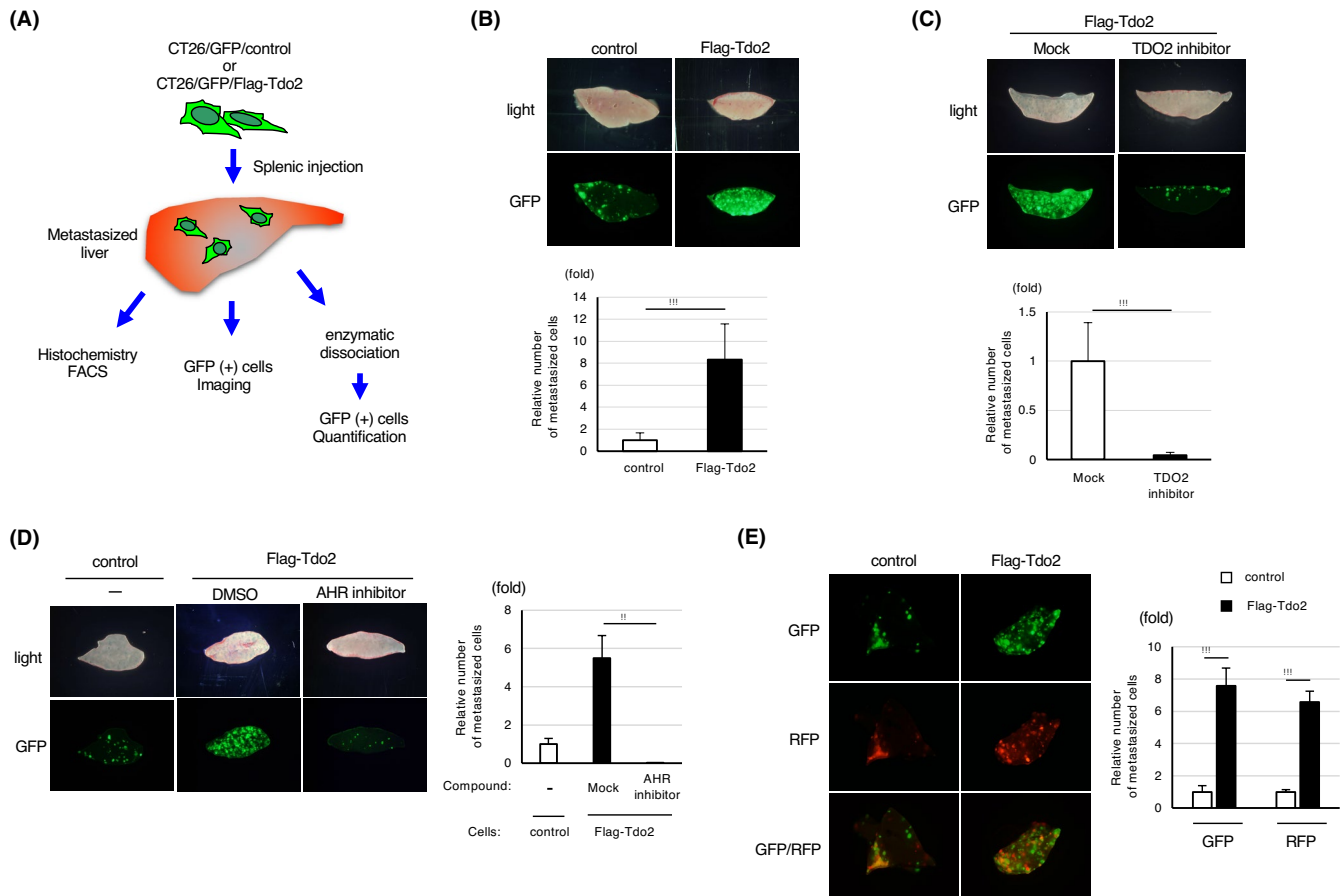


FIGURE 2 Tdo2 expression promotes liver metastasis in an Ahr-dependent manner. A, An experimental scheme of the liver metastasis analyses. B, Promotion of liver metastasis by colon cancer cells induced by Tdo2 expression. Top: Representative stereo microscopy and GFP images of the liver at 7 d after splenic injection of CT26-derived cells. Bottom: Relative numbers of liver-metastasized GFP-positive cells. C, Inhibition of Tdo2-induced liver metastasis by daily intraperitoneal injection of a Tdo2 inhibitor. Top: Representative GFP images of liver metastases. Bottom: Relative numbers of liver-metastasized GFP-positive cells. D, Inhibition of Tdo2-mediated liver metastasis by daily intraperitoneal injection of an AHR inhibitor. Left: Representative GFP images of liver metastases. Right: Relative numbers of metastasized GFP-positive cells. E, Promotion of liver metastasis by coinjected Tdo2-expressing cells. Left: Representative fluorescent images of liver metastases. Right: Relative numbers of liver-metastasized cells. Values represent the mean \pm SD; ** P < .01, *** P < .001 (two-sided Student's t -test)

is required for the inhibition of antitumor immunity and for liver metastasis stimulated by the activated Tdo2-kynurenine-Ahr pathway.

3.6 | The TDO2-AHR pathway mediates transactivation of *PD-L1* and *TDO2* in colon cancer spheroids

We next examined whether TDO2 or AHR was also required for the expression of these genes in the colon cancer spheroids that express high levels of TDO2 and kynurenine (CRC-24 M and CRC-29 M). Treatment with the inhibitors of TDO2 or AHR strongly suppressed the expression of *PD-L1* and *TDO2* as well as *IL-1b* (Figures 5A, B and S5A, B). It was previously shown that the AHR-responsive elements are located near the *PD-L1* promoter (Figure S5C),¹⁹ and ChIP analyses indeed showed that AHR was bound to the promoters of *PD-L1* and *IL-1b* (Figure S5D). In addition, in silico analyses of

genomic sequences indicated that the promoters of *TDO2* harbor a potential binding site for AHR (Figure 5C), and ChIP analyses confirmed the binding of AHR to the predicted site of the *TDO2* gene, which was abrogated in the presence of the inhibitors for TDO2 or AHR (Figure 5D). Thus, the kynurenine-AHR pathway mediates the transactivation of *PD-L1* and *TDO2* in both mouse and human colon cancer cells.

3.7 | The TDO2-AHR pathway is required for the maintenance of CSC-related characteristics in colon cancer spheroids

Remarkably, treatment with an inhibitor of TDO2 or AHR significantly inhibited the cell proliferation of the studied cancer spheroids (Figure 5E). Because the established spheroids are enriched in cells with CSC-related properties,⁴ we examined whether TDO2

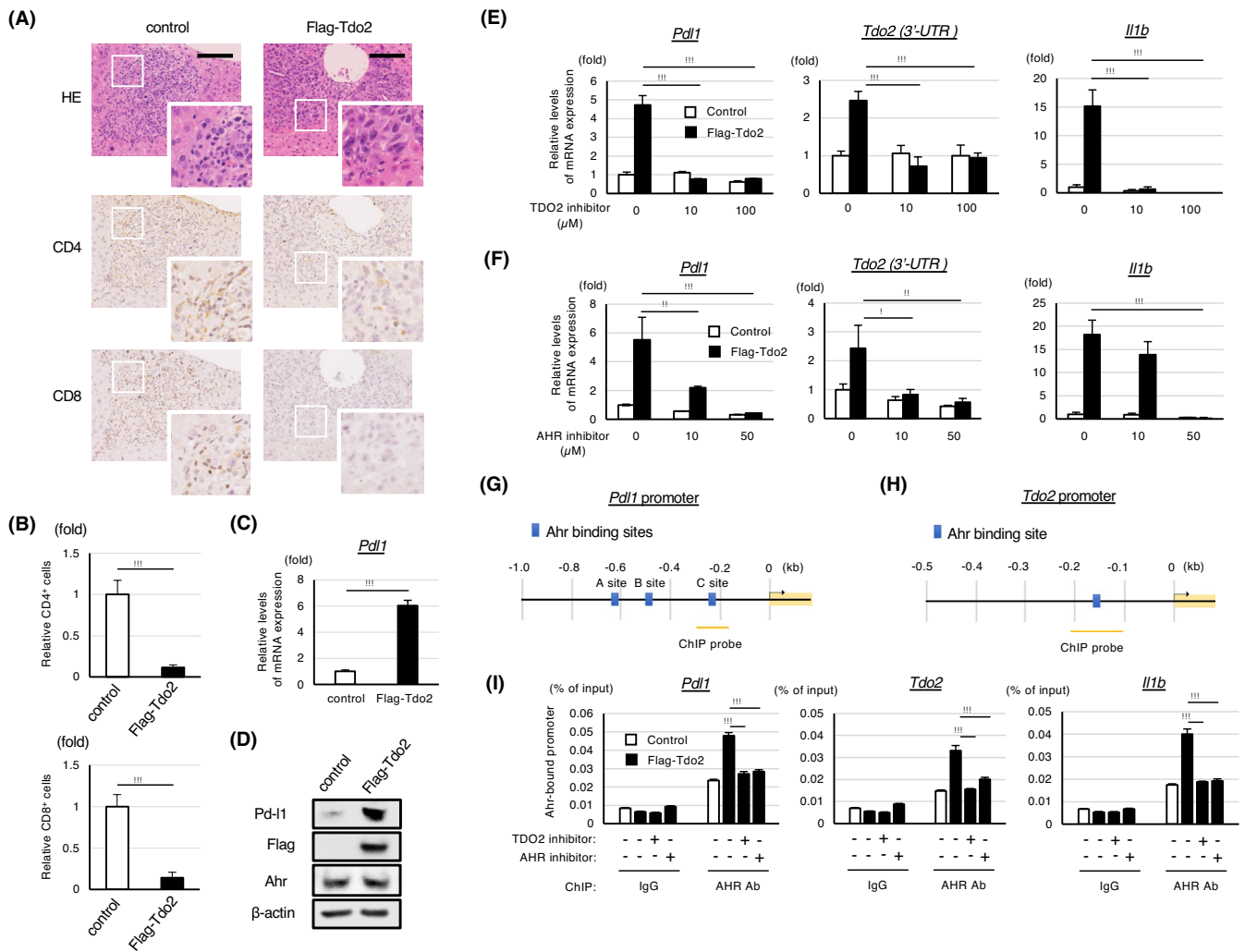


FIGURE 3 The induction of the TDO2-kynurenine-AHR pathway transactivates *Pd-1* and compromises immune responses in metastatic liver. A, H&E staining and immunohistochemical analyses of metastasized livers at 7 d after the splenic injection of the CT26/GFP cells expressing the indicated construct. Images acquired at a higher magnification are also shown. Scale bar: 100 μ m. B, Relative numbers of infiltrating CD4⁺ and CD8⁺ cells in the indicated tumors shown in (A). C, qPCR analysis of *Pd1* expression in CT26 cells expressing Flag-Tdo2 and control cells. D, Western blot analyses of the expression of the indicated proteins in the cells shown in (C). E, qPCR analyses of the expression of the indicated genes in the cells shown in (C). The cells were treated with the indicated amounts of a TDO2 or AHR inhibitor for 48 h before the analyses. For quantification of endogenous *Tdo2* expression, 3'-UTR of *Tdo2* was evaluated. G, H, Schematic representations of the predicted Ahr binding sites in the *Pd1* and *Tdo2* promoters. I, ChIP analyses of AHR binding to the indicated promoter region in CT26/GFP/Flag-Tdo2 cells or control cells. The cells were treated with the TDO2 or AHR inhibitor for 48 h or mock treated. Enrichment over input (% input) was measured by qPCR (n = 3). As for the ChIP analyses of the *Pd1* promoter, the results of the AHR binding for the C site shown in (G) are shown (the binding to the other sites was not detected). Values represent the mean \pm SD; *P < .05, **P < .01, ***P < .001

and AHR inhibitors suppressed the maintenance of stemness in the spheroids. Limiting dilution assays revealed that spheroid formation efficiency was substantially reduced by the inhibitors (Figure 5F). In addition, the expression of the colon CSC marker CD44²¹ was suppressed by the inhibition of TDO2 or AHR (Figure 5G, H). qPCR analyses indicated that the suppression of CD44 by the TDO2/AHR inhibitor was at least in part mediated at RNA levels (Figure S5E). Because it was reported that CD44 is one of the major target genes of Wnt signaling,²² we also examined whether the inhibition of Wnt signaling also suppressed CD44 expression. Of note, treatment of the spheroids with inhibitors of Wnt signaling (XAV²³ and IWR1²⁴) caused suppression of

CD44 expression at levels comparable to those observed after the treatment with the TDO2/AHR inhibitors (Figure S5E), suggesting that TDO2 and AHR are involved in CD44 regulation via the Wnt pathway.

To confirm the role of the TDO2-AHR pathway, we generated a cancer spheroid (CRC-29 M) in which the *AHR* gene was targeted for CRISPR-mediated knockout in a doxycycline-dependent manner (Figure 5I). As expected, the induction of *AHR* knockout inhibited the expression of *PD-L1* and *TDO2* as well as *IL-1b* (Figure 5J), inhibited cell proliferation (Figure 5K), and suppressed CD44 expression (Figure 5I), thus phenocopying the effects of the AHR/TDO2 inhibitors.

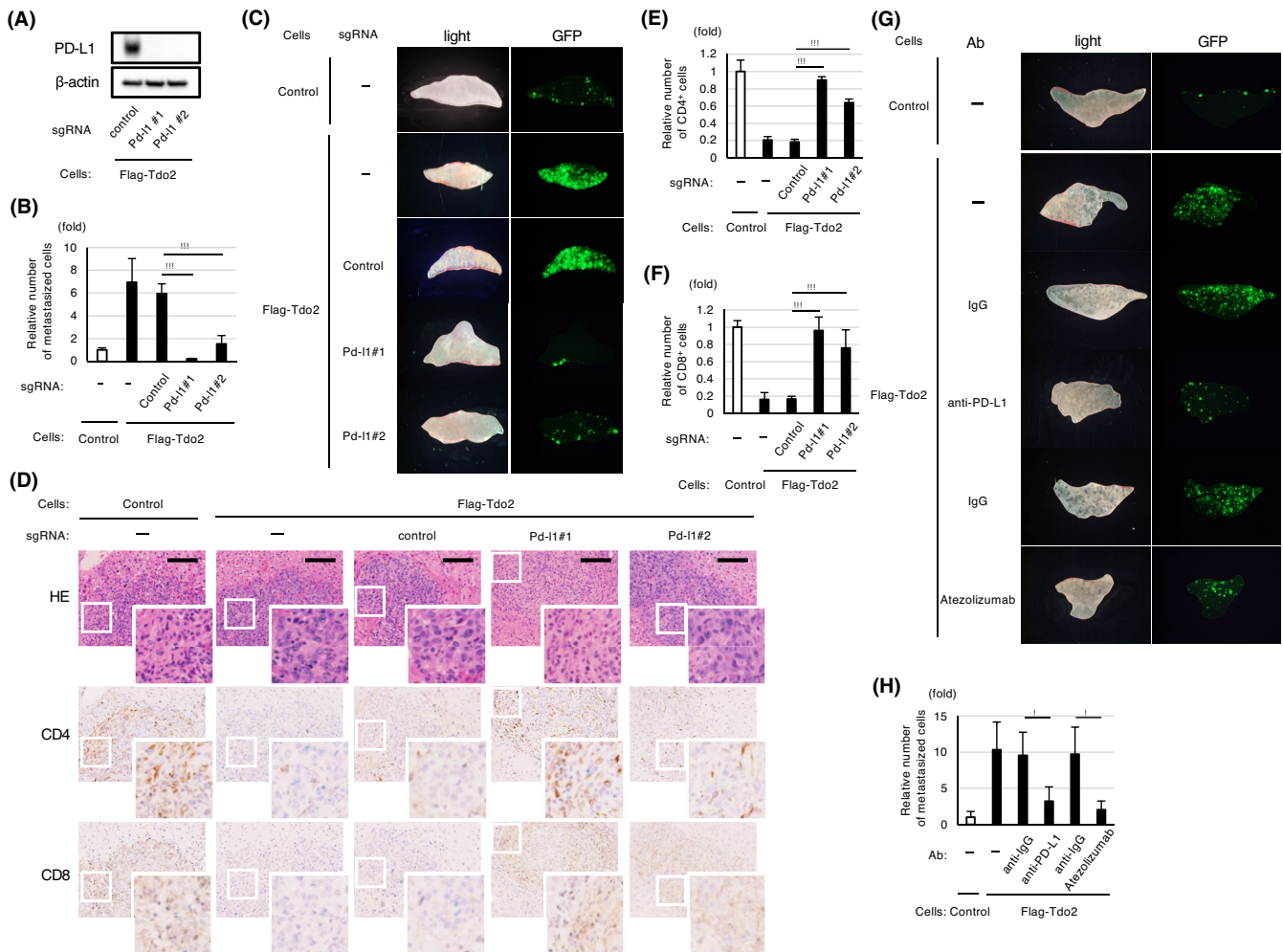


FIGURE 4 *Pd-11* is required for the Tdo2-mediated promotion of liver metastasis and suppression of immune responses. A, Western blot analyses of *Pd-11* expression in CT26 cells expressing Flag-Tdo2 subjected to *Pd-11* (*Pd-I1*#1 and #2) or control knockout. B, Abolishment of Tdo2-mediated liver metastasis by knocking out *Pd-11*. C, Representative GFP images of the liver after splenic injection of the CT26 cells shown in (B). D, Restoration of T cell accumulation in liver metastases generated by *Pd-11*-knockout cells. H&E staining and immunohistochemical analyses for the indicated lymphocytes. Scale bar: 100 μ m. E, F, Relative numbers of tumor-infiltrating CD4- and CD8-positive cells in the indicated tumors. Quantification of relative cell number was performed as described in Figure 3B ($n = 3$). G, H, Inhibition of Tdo2-mediated liver metastasis with neutralizing anti-PD-L1 antibodies after splenic injection of the indicated CT26 cells. CT26/Flag-Tdo2 cells were incubated with the indicated antibodies for 48 h and then used for splenic injection. G, Representative GFP images of the liver after splenic injection. H, The extent of inhibition determined by measuring the relative numbers of liver-metastasized CT26 cells. Values represent the mean \pm SD; * $P < .05$, ** $P < .01$, *** $P < .001$

3.8 | The TDO2-AHR pathway directly activates *LGR5* and mediates the maintenance of Wnt signaling in colon cancer spheroids

Because the Wnt signaling pathway plays a pivotal role in colon cancer stemness,²⁵ we examined the effect of the TDO2/AHR inhibitors on the expression of representative Wnt target genes. Inhibition of TDO2 or AHR suppressed the expression of all the Wnt target genes we evaluated (Figures 6A and S6A) and blocked nuclear localization of β -catenin (Figures 6B and S6B). In accordance, the inducible knockout of AHR suppressed the expression of the Wnt target genes and nuclear localization of β -catenin

(Figure 6C, D). Collectively, the TDO2-kynurenine-AHR pathway mediates the activation of the Wnt signaling pathway and cancer stemness.

Of the six Wnt target genes regulated by TDO2/AHR, we further investigated whether *LGR5* is directly regulated by AHR. In silico analyses showed that the promoter regions of the *LGR5* gene harbors potential binding sites for AHR (Figures S3G and S6C). ChIP analyses confirmed that AHR was bound to the *LGR5* promoter, and the binding was abolished in the presence of the inhibitors of AHR or TDO2 (Figure S6D). Thus, the TDO2-AHR pathway directly regulates *LGR5* expression, which is likely to contribute to Wnt regulation and the stemness of colon CSCs.

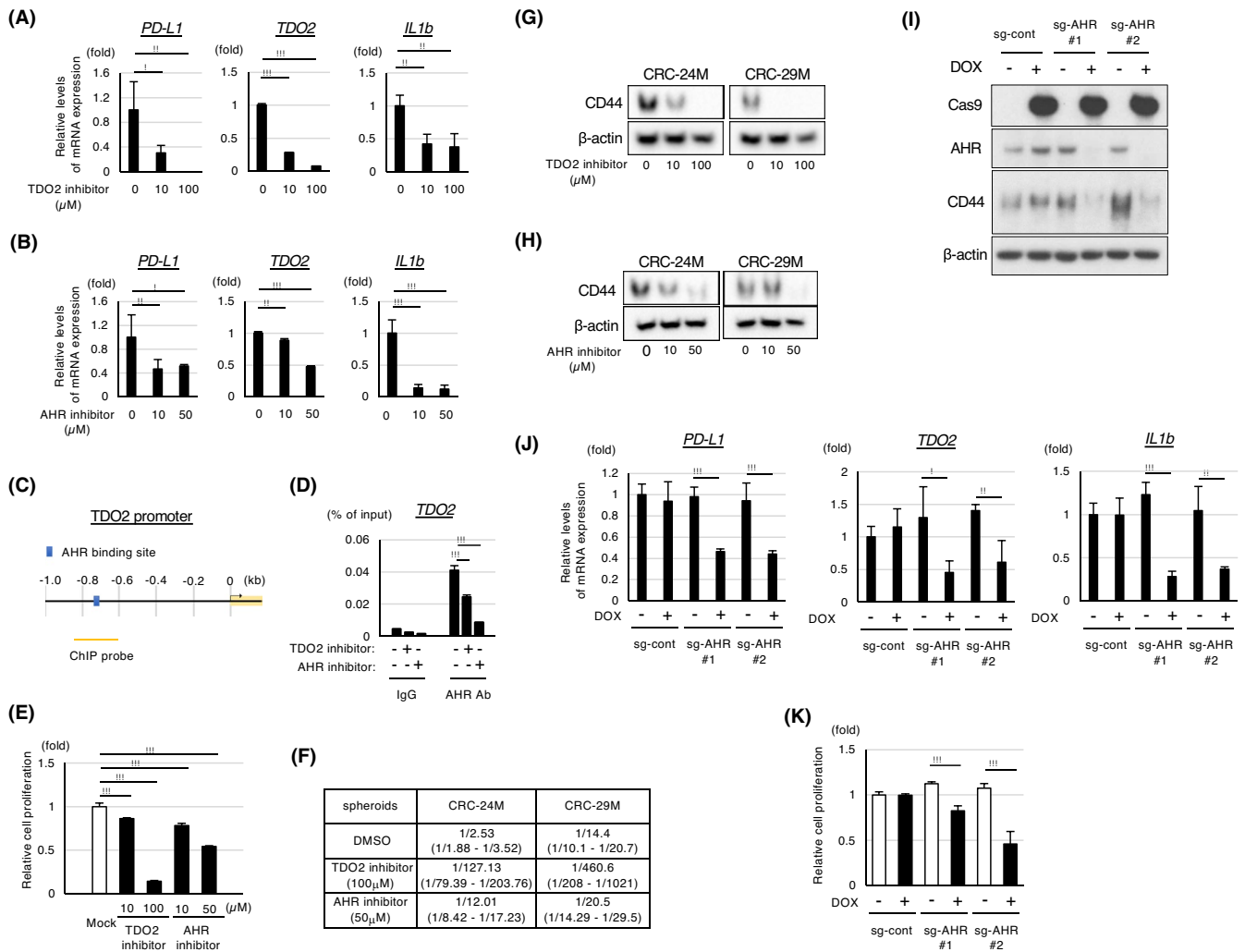


FIGURE 5 The TDO2-AHR pathway is required for the transactivation of *PD-L1* and *TDO2* and for the maintenance of stemness in colon cancer spheroids. A, B, qPCR analyses of *PD-L1*, *TDO2*, and *IL1b* expression in cancer spheroids (CRC-29 M) treated with the indicated concentrations of an inhibitor of TDO2 (A) or AHR (B) for 96 h. C, Schematic representation of predicted AHR-binding sites in the promoters of *TDO2*. D, ChIP analyses of the AHR-bound promoters of the *TDO2* gene in spheroid cells (CRC-29 M) treated with the TDO2 inhibitor (100 μ M) or AHR inhibitor (50 μ M) for 96 h. E, Relative proliferation of spheroid cells (CRC-29 M) measured by CellTiter-Glo assays ($n = 4$). The spheroids were treated with the indicated concentrations of the TDO2 or AHR inhibitor for 7 d. F, Limiting dilution assay with FACS-sorted spheroid cells. An average frequency of self-renewing cells (upper lanes) and confidence interval (lower lanes) are shown. G, H, Western blot analyses of CD44 protein expression in the indicated spheroid cells treated with the inhibitor of TDO2 (G) or AHR (H) for 96 h. I, Western blot analyses in spheroid cells (CRC-29 M) introduced with doxycycline-inducible Cas9 and the indicated sg-RNA in the presence or absence of 1 μ M doxycycline for 5 d. J, qPCR analyses of the indicated genes in the spheroids shown in (I). K, Relative proliferation of spheroid cells shown in (I). Values represent the mean \pm SD; * $P < .05$, ** $P < .01$, *** $P < .001$

3.9 | *TDO2* is coexpressed with *PD-L1* and *LGR5* in human colon tumors in vivo

To examine whether *TDO2* expression is associated with cancer stemness and *PD-L1* expression in vivo, we investigated the coexpression of *TDO2* with *LGR5* and *PD-L1* in a mouse xenograft model. Metastatic spheroid cells that express relatively high levels of *TDO2* (CRC-24 M or CRC-29 M) or nonmetastatic spheroid cells that express *TDO2* at marginal levels (CRC-19 or CRC-20) were subcutaneously injected into NOD/SCID mice to generate xenograft tumors, and colocalization of *TDO2* with *LGR5* or *PD-L1* in the formed tumors was examined by in situ hybridization. As expected, the proportion

of cancer cells with high *TDO2* levels (≥ 2 dots) was higher in the xenograft tumors expressing detectable *TDO2* (CRC-24 M or CRC-29 M) than in those not (CRC-19 or CRC-20) (Figures 7A, B and S7A, B). The *TDO2* expression levels in the former xenografted tumor cells were positively correlated with the *LGR5* levels (Figures 7C and S7C). Likewise, *TDO2* expression in tumor cells was positively correlated with *PD-L1* expression (Figures 7D and S7D).

We also examined the coexpression of *TDO2* with *PD-L1* or activated β -catenin in the xenografted tumor. In accordance with the results of the in situ hybridization, *TDO2* and *PD-L1* were coexpressed in metastatic spheroid-derived xenograft tumors, and the expression levels of these proteins in the metastatic spheroid-derived tumors

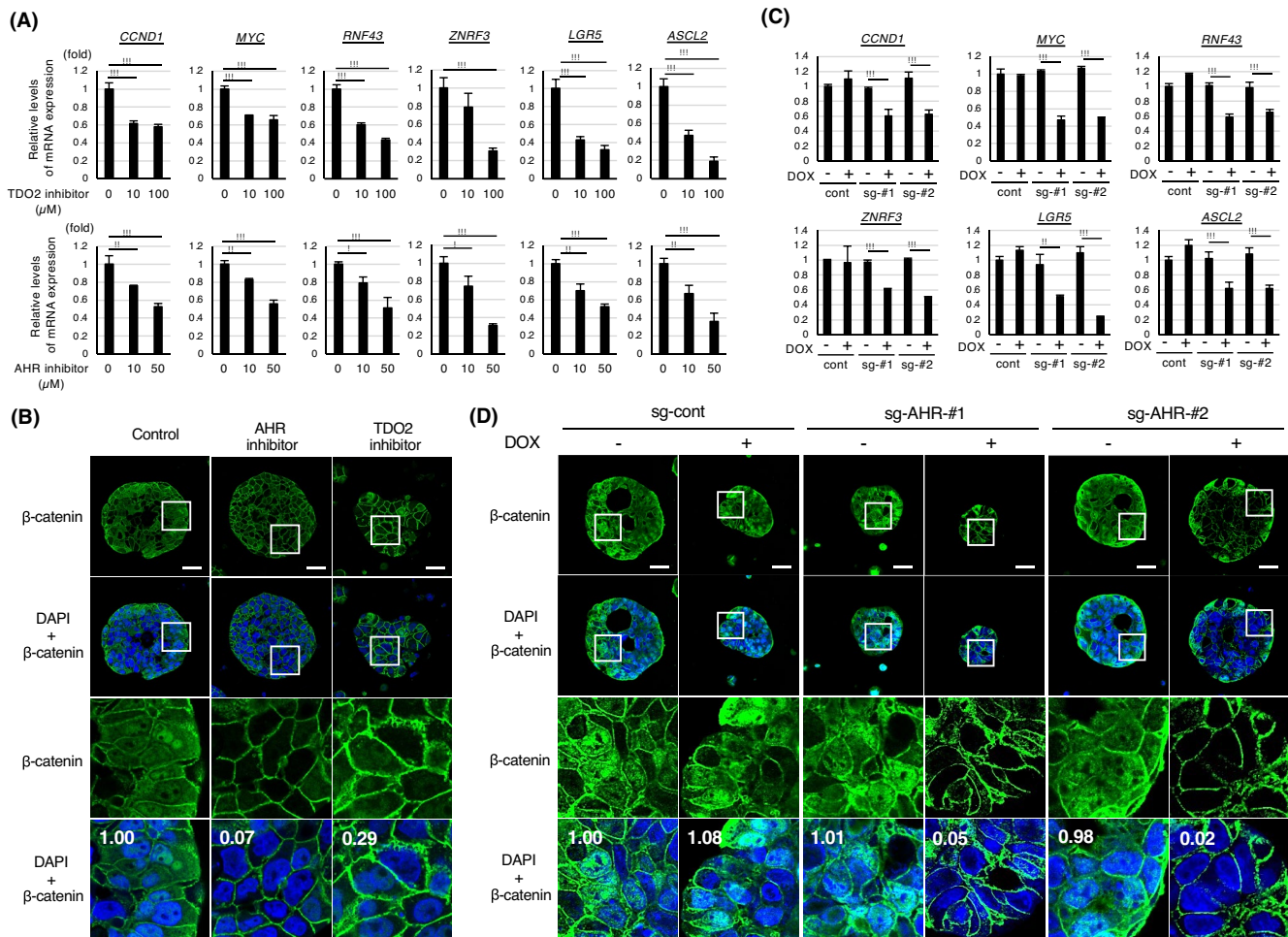


FIGURE 6 The TDO2-AHR pathway mediates the maintenance of Wnt signaling in colon cancer spheroids. A, qPCR analyses of representative Wnt target genes in the metastatic spheroids (CRC-24 M) after treatment with the indicated concentrations of the inhibitor of TDO2 or AHR for 96 h. B, Immunostaining of the spheroids (CRC-24 M) with β -catenin in the presence of the indicated inhibitor for 96 h. Nuclei were counterstained with DAPI. Relative quantification values of nuclear β -catenin were shown in the bottom columns. Scale bar: 25 μ m. C, qPCR analyses of representative Wnt target genes in the AHR- or TDO2-inhibited spheroids shown in Figure 5G (100 μ mol/L). D, Immunostaining of the spheroids shown in Figure 5I. Relative quantification values of nuclear β -catenin were shown in the bottom columns. Scale bar: 25 μ m. Values represent the mean \pm SD; * P < .05, ** P < .01, *** P < .001

were significantly higher than those in the nonmetastatic ones (Figures 7E and S7E). In addition, nuclear β -catenin staining was observed in the metastatic spheroid-derived tumors, and coexpressed with TDO2 (Figures 7F and S7E). Collectively, these data demonstrate that TDO2 was coexpressed with LGR5, PD-L1, and nuclear β -catenin in xenografted tumors derived from metastatic spheroids.

3.10 | TDO2 expression is positively associated with PD-L1 expression in clinical specimens

We next examined whether TDO2 expression is associated with PD-L1 expression using surgical specimens of colon cancers. A positive correlation was observed between the expression levels of TDO2 and PD-L1 in clinical colon cancer samples from The Cancer Genome Atlas (TCGA) database (Figure 7G). Further, immunohistological analysis of the samples for each stage of colon cancer showed that

TDO2 expression levels were increased in advanced stages of colon cancer (Figure 7H). Remarkably, coimmunostaining studies of the clinical specimens revealed that TDO2 and PD-L1 were coexpressed in metastatic colon cancer (Figures 7I and S7G), supporting the positive role of TDO2-induced PD-L1 expression during metastatic processes.

4 | DISCUSSION

It was previously reported that the kynurenine-AHR pathway plays prometastatic roles in several cancers including breast cancer^{26,27} and squamous cancers.^{28,29} The AHR activation may play roles in evasion from immunosurveillance, as the AHR-mediated transactivation of PD-L1 is induced by benzo(a)pyrene, an exogenous tobacco carcinogen.¹⁹ In this paper, we extended the prometastatic roles of the AHR pathway by showing that the TDO2-induced kynurenine

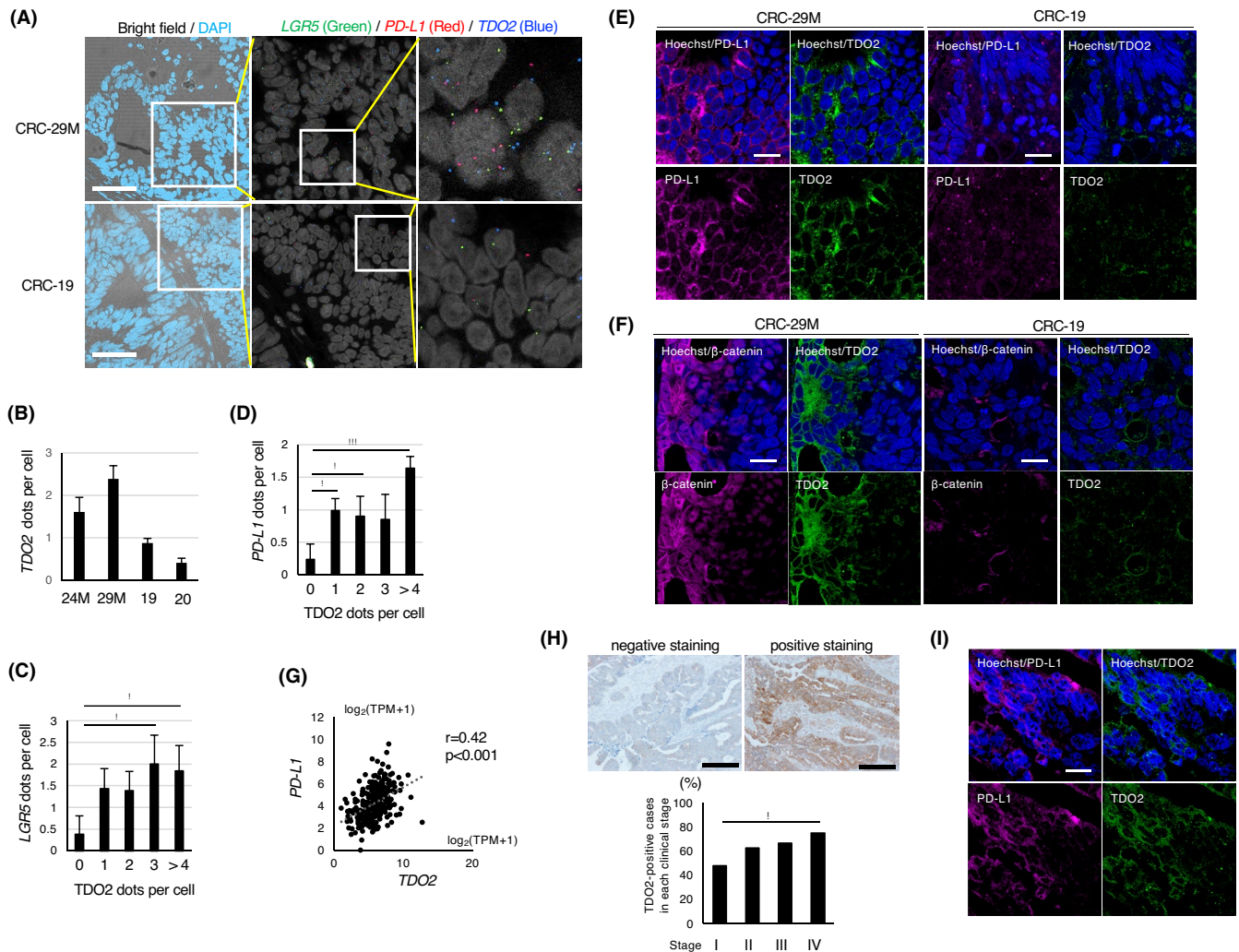


FIGURE 7 TDO2 is coexpressed with LGR5 and PD-L1 in colon tumors and positively associated with PD-L1 expression in clinical specimens. A, In situ RNA hybridization of xenograft tumors derived from cancer spheroids. Left: Merged images of bright-field microscopy and DAPI staining of the tumors derived from CRC-29 M and CRC-19. Scale bar: 50 μ m. Middle and right: In situ RNA hybridization with LGR5 (green), PD-L1 (red), and TDO2 (blue) probes. Images acquired at a higher magnification are shown on the right. B, Average numbers of TDO2-hybridizing dots per cells in the indicated xenograft tumors. C, D, Correlation of the average number of hybridizing dots for (C) LGR5 or (D) PD-L1 with the indicated number of dots for TDO2 in xenografted tumors (CRC-29 M). E, Representative coimmunostaining of xenograft tumors (CRC-29 M and CRC-19) for PD-L1 and TDO2. Scale bar: 10 μ m. F, Representative coimmunostaining of xenograft tumors (CRC-29 M and CRC-19) for β -catenin and TDO2. Scale bar: 10 μ m. G, Positive association between PD-L1 and TDO2 mRNA expression in clinical specimens of colon cancer from The Cancer Genome Atlas (TCGA) database. H, Immunohistochemical analyses of TDO2 in clinical specimens of human colon cancers. Top: Representative images of negative and positive staining. Scale bar: 400 μ m. Bottom: Proportion of TDO2-positive cases in each clinical stage is shown. I, Representative coimmunostaining of surgical specimens of primary colon cancer of patients with liver metastasis. Scale bar: 10 μ m. Values represent the mean \pm SD; * P < .05, ** P < .01, *** P < .001

production and the resulting activation of the AHR pathway facilitate liver metastasis by stimulating PD-L1-mediated inhibition of anticancer immunity and Wnt signaling-associated cancer stemness. Thus, kynurenine production by TDO2, together with various endogenous and exogenous ligands, may converge on AHR-mediated activation of PD-L1, possibly affecting cancer immunosurveillance. We also revealed LGR5, TDO2, and PD-L1 as direct AHR targets that are likely to mediate these phenotypes.

Overall, our data suggest that TDO2-mediated AHR activation facilitates liver metastasis via generation of immune-evasive CSCs (Figure S7H). Consistent with our model, accumulating reports

suggested that CSCs can evade host immunosurveillance.³⁰⁻³⁶ The proposed model suggests that targeting the TDO2-AHR pathway in the metastatic CSCs could be a therapeutic option to block liver metastasis. By blocking of this pathway, both cancer stemness and immune-evasive capability might be targeted, giving an advantage over other CSC-targeted therapies.

While our data indicate that the TDO2-AHR pathway facilitates liver metastasis, induction of stemness-related genes may be context dependent. The regulation of PD-L1, TDO2, and IL1b by the TDO2-AHR pathway was observed in both CT26 cells and human cancer spheroids, whereas Lgr5 expression was not detectable in

CT26 cells (data not shown). Presently, in CT26 cells, it is not clear whether the TDO2-AHR pathway regulates stemness-related characteristics, including activation of the Wnt signaling or CD44-related pathway. Thus, the promotion of cancer stemness by the TDO2-AHR may be cell type dependent.

Because the AHR pathway is known to play positive roles in cancer progression, IDO1/TDO2-mediated kynurenine production and the resulting activation of AHR are regarded as potential therapeutic targets.^{37,38} Especially, IDO1 has been a major focus of therapeutic intervention due to its widespread expression in cancer.^{39,40} Although recent clinical trials against IDO1 did not result in clear inhibition of primary cancers,⁴¹ the lack of response may be caused by resilient AHR activity induced by TDO2-derived kynurenine, suggesting that TDO2 may need to be simultaneously targeted for effective therapy.

Our data revealed that TDO2-activated AHR targets the TDO2 gene for transactivation, suggesting that transient activation of AHR can trigger the formation of a positive feedback loop with TDO2. This suggests that, once temporally activated by a variety of ligands or tryptophan derivatives, AHR may initiate the positive feedback loop between AHR and TDO2. Interestingly, AHR has been reported to transactivate IDO1 and AHR itself in undifferentiated ES cells,¹⁰ suggesting the existence of multiple feedback loops activating AHR.

While some metastatic spheroids expressed high levels of *TDO2* and kynurenine, others expressed them at relatively low levels (Figure 1C), suggesting that other unknown mechanisms may substitute the activation of the *TDO2*-kynurenine pathway in metastatic colon cancers that express low levels of *TDO2*-kynurenine. It may be compensated by kynurenine-independent mechanisms of AHR activation via other endogenous and exogenous ligands. Alternatively, the dependence on the *TDO2*-kynurenine pathway for CSC maintenance may be influenced by the extent of pre-existing activation of the Wnt signaling pathway, and the cells totally depend on the activation of the *TDO2*-kynurenine-AHR pathway for Wnt activation in the absence of the mutation in the Wnt-related pathway. In any case, future investigation warrants a global picture of Wnt activation in metastatic colon cancers.

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DISCLOSURE

The authors declare no competing interests.

ACCESSION NUMBERS

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SUPPORTING INFORMATION

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