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Original Article

IL4I1 and tryptophan metabolites enhance AHR signals to facilitate colorectal cancer progression and immunosuppression

Heng Sun[1*], Wenjie Han[2*], Juyi Wen[1], Xue Ma[2]

[1]*Senior Department of Oncology, The Fifth Medical Center of Chinese PLA General Hospital, Beijing, China;*
[2]*Department of Geriatric Medicine, The Sixth Medical Center of Chinese PLA General Hospital, Beijing, China.*
[*]*Equal contributors.*

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Abstract: Objective: The molecular mechanisms underlying tumor progression and drug resistance in colorectal cancer remain to be fully understood. Recent studies have reported a pro-tumorigenic role of an amino acid oxidase named interleukin-4-induced-1 (IL4I1). Here, we investigate the role and molecular mechanism of IL4I1 in colorectal cancer. Methods: We employed bioinformatics analysis and experimental validation by using clinical samples and a variety of cell-based assays, including western blot, Transwell assay, patient-derived organoid culture, Immunofluorescence assay, T cell cytotoxicity assay, and flow cytometry. Results: Bioinformatics analysis showed a higher IL4I1 expression in colorectal cancer tissues than in normal tissues. In vitro overexpression of IL4I1 enhanced the proliferation, migration, and invasion of colorectal cancer cells. In addition, deprivation of Tryptophan (Trp) in cultural medium diminished the oncogenic effect of IL4I1. Furthermore, we observed a positive correlation of IL4I1 and AHR expression in the TCGA database of colorectal cancer. We also detected an enhanced cytoplasmic expression and nuclear translocation of Aryl hydrocarbon receptor (AHR). Moreover, IL4I1 overexpression suppressed the cytolytic killing of tumor cells and enhanced T cell exhaustion. Finally, in the organoid culture model, we found that immunotherapy and SR-1 combination treatment could induce higher level of apoptosis than did the immunotherapy or SR-1 treatment alone. Conclusion: we demonstrated that IL4I1 facilitated colorectal cancer progression and immunosuppression through tryptophan metabolism dependent on AHR activation.

Keywords: IL4I1, pro-tumorigenic, colorectal cancer, AHR, T cell exhaustion, immunosuppression

Introduction

Colorectal cancer is the most commonly diagnosed cancer and the leading cause of cancer-related death according to the 2018 global cancer statistics [1]. Although chemotherapy has been widely used in treating colorectal cancer, a large proportion of patients display resistance to chemotherapy [2, 3]. Therefore, understanding the molecular mechanisms involved in disease progression as well as drug resistance will help exploit promising therapeutic targets to improve the survival of patients with colorectal cancer.

Accumulating evidence has shown that alterations in tumor cell metabolism attribute to tumor progression and drug resistance through shaping tumor microenvironment [4, 5]. Multiple metabolic pathways such as PI3K-Akt-mTOR, glutamine metabolism, and lipid metabolism have been reported to be involved in the tumor development and drug resistance [6-8]. In particular, recent findings have identified the essential amino acid tryptophan (Trp) catabolism as an important player in inducing an immunosuppressive tumor microenvironment [9, 10]. Aryl hydrocarbon receptor (AHR), a transcription factor activated by various ligands including the metabolites of Trp, promotes tumorigenesis and chemotherapy resistance in several cancers [11]. AHR not only increases the proliferation and migration of cancer cells but also directly induces cancer immune tolerance [12].

Interleukin-4-induced gene 1 (IL4I1) is a member of the L-amino-acid oxidase (LAAO) family

and catalyzes the oxidative deamination of phenylalanine into phenylpyruvate [13]. Recently, IL4I1 has been found to catalyze L-tryptophan and promote the Inhibition of T cell proliferation [13]. Furthermore, in a chronic lymphocytic leukemia murine model, IL4I1 catalyzed Trp to activate AHR and promoted tumor progression [14]. Nevertheless, the expression and function of IL4I1 in colorectal cancer remains unclear. Therefore, in the present study, we investigated the effect of IL4I1 on the oncogenesis of colorectal cancer and found that IL4I1 promoted tumorigenesis, progression, immune evasion as well as chemotherapy and immune checkpoint therapy resistance through activating AHR, indicating the oncogenic role of IL4I1 in colorectal cancer.

Materials and methods

Cell culture and transfection

Human colorectal cancer cell line HCT116 and murine colorectal cancer cell line MC38 were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Gibco, CA, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, CA, USA) in a humidified atmosphere containing 5% CO_2 at 37°C. Cell transfection was performed by using Lipofect8000 (Beyotime, C0533, China). IL4I1 mammalian expression plasmid was constructed by cloning IL4I1 into PCDNA3.1 vector. IL4I1 was knocked out in cells by using CRISPR-Cas9. AHR was knocked down in CD8+ cells by using shRNA.

MTT assay

MTT assay was performed following the manufacturer's instruction. Briefly, MC38 and HCT116 cells (5000-cell number/well) were seeded in 96-well plates overnight, and then 20 μL of MTT solution (5 mg/mL, Sigma-Aldrich, USA) was added to each well for 4 h. Before measurement, 150 μL of DMSO (Sigma-Aldrich) was added into each well, and the absorbance at 490 nm was measured with a microplate reader (Thermo Fisher Scientific, USA).

Western blot

Western blot analysis was conducted following the standard protocol. Briefly, MC38 and HCT116 cells were collected and lysed in RIPA lysing buffer (Beyotime, P0013C, China) containing protease and phosphatase Inhibitor cocktails (Beyotime, P1010, China). Cell lysates were quantified by BCA Protein Assay kit (Beyotime, P0010, China), and 20 μg proteins were subjected to SDS-PAGE before transferred onto nitrocellulose membrane (Corning, USA). The membranes were blocked with 5% bovine serum albumin and incubated with specific primary antibodies. The primary Antibodies (Abs) used in our study were: IL4I1 (PA5-106825, Invitrogen), Actin or GAPDH as loading control? An HRP-conjugated secondary antibody (Beyotime, A0239, China) was used to detect the protein-antibody complex, and the signal was subsequently visualized by chemiluminescence. The images were captured with western blotting imager system (Tanon 4800, China).

Colony formation assay

Cells were seeded into a 6-well plate at a density of 500 cells per well and cultured for two weeks. Then, the cell colonies were fixed with 4% paraformaldehyde Fix Solution (Beyotime, P0099, China) for 15 min and was stained with 0.5% crystal violet for 5 min (Beyotime, C0121, China). After air dry, the culture dishes were examined, and the number of colonies was counted and analyzed by Image J.

Patient derived organoid (PDO) culture

Fresh colon cancer tissues were collected from patients at the Fifth Medical Center of Chinese PLA General Hospital. Written informed consent from was obtained from each patient before surgery. This study was approved by the Clinical Research Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital. Patient-derived tumor tissues were washed with HBSS, cut into 2 mm pieces, and centrifuged at 800 rpm for 5 min. Subsequently, the supernatants were digested in by 2 mg/mL collagenase IV (Gibco) and 0.2 mg/mL DNase I (Sigma-Aldrich) for 30 min, followed by centrifugation at 800 rpm for 5 min. The cell pellet was resuspended with HBSS to obtain single tumor cell suspension. Next, the single tumor cell was mixed with Matrigel (Corning) and cultured for the formation of patient derived organoids (PDOs). The PDO culture medium consisted of the following agents: advanced DMEM/F-12 (Gibco) supplemented

with B-27 (1:50, Gibco), N-2 (1:100, Gibco), HEPES (1:1000, Gibco), penicillin (1:100, Solarbio), nicotinamide (10 mM, Sigma-Aldrich), N-acetyl-l-cysteine (1.25 mM, Sigma-Aldrich), human EGF (50 ng/mL, PeproTech), R-spondin 1 (100 ng/mL, PeproTech), noggin (100 ng/mL, PeproTech), and Wnt3a (100 ng/mL, Fitzgerald).

Transwell assay

Transwell assay was conducted in 24-well chambers (Corning Glass Works, Corning, N.Y., USA). Briefly, cells (1×10^5) in 200 μL serum-free medium were added to the upper chamber coated with Matrigel, whereas the complete medium containing 20% FBS was added to the lower chamber. The cells were cultured for 24 h, and the migrated cells fixed with 95% alcohol and stained with crystal violet solution. Finally, the stained migrated cells in five different visual fields were examined and counted under the microscope.

Immunofluorescence

For immunofluorescence staining, slides containing cultured cells or tissue sections from mice were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked with 4% bovine serum albumin in TBST for 30 min. The cells or tissue were incubated with anti-AHR antibody (MA1-513, Invitrogen) at 4°C overnight. After extensive wash with PBS, slides were incubated with fluorescent dye conjugate secondary antibodies for 1 h. The images were acquired with a fluorescence microscope.

Immunohistochemistry (IHC) staining

IHC staining was carried out following the standard protocol. Briefly, the formalin-fixed paraffin-embedded tissue sections were de-paraffinized and rehydrated. After incubated in heated antigen retrieval solution, the slides were incubated in 3% methanolic H_2O_2 for 30 min and treated with 0.5% triton X-100 for 30 min. The slides were blocked with 10% goat serum and incubated with anti-IL4I1 antibody (PA5-113266, Invitrogen) at 4°C overnight. After extensive wash, the slides were incubated with appropriate secondary antibody for 1 h. DAB-based detection was used with Ultravision Quanto Detection system (ThermoScientific, Waltham, MA). The slides were counterstained with hematoxylin, dehydrated, cleared, and mounted with DPX. The images were photographed using the microscope.

Apoptosis assay

Annexin V-FITC/7AAD apoptosis assay was used to measure the killing of target cells. Vector control- or shAHR-expressing cytotoxic T lymphocyte (CTL) cells were co-cultured with PDOs at a 5:1 (effector:target) ratio. Annexin V-FITC/7AAD signal was measured at 24 h after co-culture.

Flow cytometry

For cell surface staining, viability staining with Zombie Violet™ Fixable Viability Kit (Biolegend, USA) was performed in CD8 T cells following the manufacturer's protocol. Cell surface markers used were: anti-CD8 (Biolegend, USA), anti-PD-1 (Biolegend, USA), anti-TIM3 (Biolegend, USA), and anti-CD45 (Biolegend, USA). For intracellular cytokine staining, cells were first fixed with Fixation Buffer (MultiSciences, China) for 20 min on ice and permeabilized with Permeabilization Buffer (MultiSciences, China). Then, cells were stained with anti-TNFα (Biolegend, USA) and anti-IFNγ (Biolegend, USA) for 30 min on ice. The stained cell samples were resuspended in PBS and analyzed in a CytoFLEX Platform (Beckman, USA).

Lentiviral production

293T cells were cultured in a T25 flask overnight before transfection. Cells were co-transfected with lentiviral vector of PLKO.1 control or indicated plasmids (PLKO.1-BFP-shAHR) (Obio, China) and lentiviral packaging plasmids (pMD2.G and pspAX2). The production and collection of lentiviruses were carried out as previously described [15].

Lentiviral transduction and maintenance of primary human CTL cells

Briefly, tumor-infiltrating lymphocytes were isolated using EasySep™ human CD8+ T Cell Isolation Kit (STEMCELL, Canada) according to the manufacturer's instruction and plated in 96-well plates. The purified CD8+ T cells were then stimulated with anti-CD3 (Biolegend, USA),

anti-CD28 (Biolegend, USA) (1 μg/μL), and recombinant human IL-2 (Peprotech, Israel) overnight before T cells were infected with shAHR-expressing lentiviral particles. The percentage of infected cells was assessed 2 days after infection by flow cytometry to detect the expression of BFP. The infected human T cells could be kept in culture and used for in vitro assays for 2 weeks.

Intracellular cytokine staining

To assess intracellular cytokine production, 10 ng/mL of phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL of ionomycin or 10 μM of CEA peptide were added to plated splenocytes or tumor-infiltrating lymphocytes in a 96-well plate. After treatment for 30 min, GolgiStop (monensin; BD 554724) was added to the wells at a 1/1,500 dilution, and the cells were cultured for an additional 4 h before surface staining and intracellular staining. Fixation and permeabilization for the intracellular detection of cytokines were performed with the BD kit (554714) according to the manufacturer's instruction.

Bioinformatic analyses

The expression of IL4I1 in tumor and normal tissue was compared on the GEPIA2 website, which contained gene expression data of TCGA and GTEx databases [16]. The co-expressed genes were screened using the Pearson method.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 8 or SPSS version 19. The experimental data were presented as mean ± SD, and all experiments were independently repeated a minimum of three times.

Results

IL4I1 promoted tumor progression in colorectal cancer

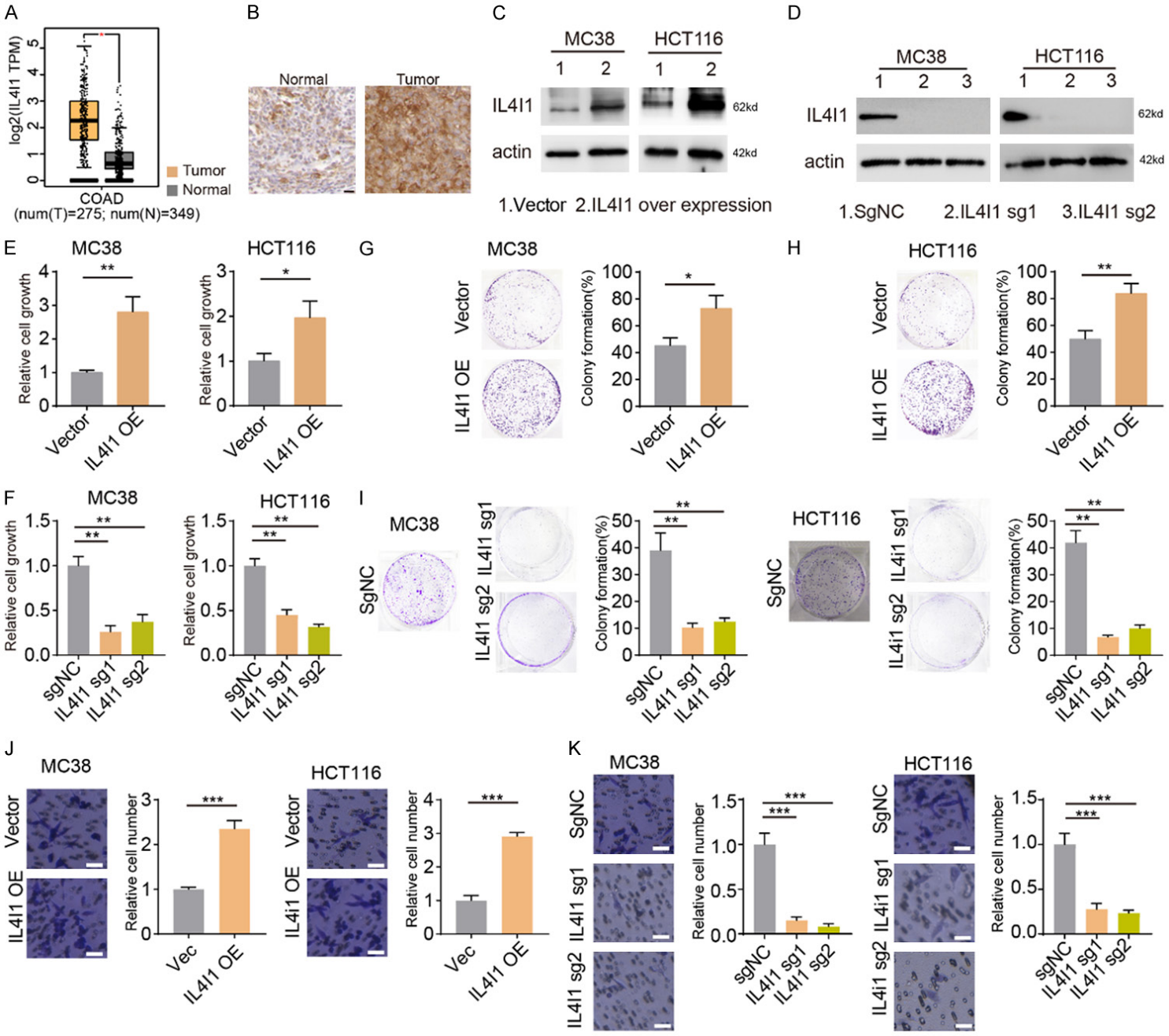
To investigate the role of IL4I1 in tumorigenesis, we first analyzed *IL4I1* gene expression by using TCGA database and found an elevated *IL4I1* expression in colorectal cancer samples compared with normal tissues (**Figure 1A**). Importantly, this elevated IL4I1 expression was validated by Immunohistochemistry staining of the tumor samples we collected (**Figure 1B**). To directly determine the role of IL4I1 in colorectal cancer, we examined the effect of altered IL4I1 expression on the growth of colorectal cancer cells. IL4I1 was either overexpressed or knocked out by sg IL4I1 in MC38 cells and HCT116 cells, and the successful IL4I1 overexpression or knockout was verified by western blot (**Figure 1C**, **1D**). We found that overexpression of IL4I1 significantly enhanced the proliferation and colony formation of MC38 and HCT116 cells (**Figure 1E**, **1G**, **1H**), while knockdown of IL4I1 markedly suppressed these effects (**Figure 1F**, **1I**). Moreover, we employed Transwell assays to determine the effect of altered IL4I1 expression on the motility of cells and found an enhanced cell invasion in IL4I1 overexpressed MC38 and HCT116 cells (**Figure 1J**), whereas knockdown of IL4I1 suppressed cell invasion (**Figure 1K**). Together, these results indicated that IL4I1 promoted the growth and invasion of colorectal cancer cells.

IL4I1 promoted colorectal cancer tumorigenesis and metastasis through tryptophan metabolism pathway

To explore the molecular mechanisms by which IL4I1 promotes tumorigenesis, we investigated whether IL4I1 promoted colorectal cancer progression dependent on Trp metabolism. For this purpose, we used Trp-free medium for MC38 and HCT116 cell culture and found that the IL4I1 overexpression-induced growth and colony formation of MC38 and HCT116 cells was attenuated when cells were cultured in Trp free medium (**Figure 2A-C**). Similarly, the enhanced migration ability in IL4I1 overexpressed MC38 and HCT116 cell lines was diminished when the cultural medium was deprived of Trp (**Figure 2D**, **2E**), suggesting that Trp metabolism pathway was required for IL4I1-promoted colorectal cancer progression.

IL4I1 enhanced the nuclear translocation of AHR and exerted its effects dependent on the activation of AHR

To further explore whether the metabolites of Trp catalyzed by IL4I1 were involved in colorectal cancer progression through activating AHR signaling, we first analyzed the association on the expression between *IL4I1* with *AHR* by using TCGA data and found a positive correla-


A
log2(IL4I1 TPM)
Tumor
Normal
COAD
(num(T)=275; num(N)=349)
B
Normal
Tumor
C
MC38
HCT116
1 2 1 2
IL4I1
actin
62kd
42kd
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D
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Relative cell number
sgNC
IL4I1 sg1
IL4I1 sg2

Figure 1. IL4I1 overexpression promoted the proliferation and migration of colorectal cancer cells. (A) IL4I1 expression between normal and tumor tissue based on TCGA cohort. (B) Immunohistochemistry analysis of IL4I1 in normal and colorectal cancer tissues from patients. Bar = 50 μm. (C, D) Western blot analysis of IL4I1 overexpression or depletion in MC38 (C) and HCT116 cells (D). (E) MTT assay of the vector- and IL4I1-overexpressing MC38 and HCT116 cells. (F) MTT assay of the sgNC- and sgIL4I1-expressing MC38 and HCT116 cells. (G, H) Colony formation assay of the vector- and IL4I1-overexpressing MC38 cells (G) and HCT116 (H). (I) Colony formation assay of the sgNC- and sgIL4I1-expressing MC38 or HCT116 cells. (J) The relative numbers of the migrated vector- and IL4I1-overexpressing MC38 or HCT116 cells. Bar = 40 μm. (K) The relative numbers of the migrated sgNC- and sgIL4I1-expressing MC38 or HCT116 cells. Bar = 40 μm. The data were presented as means ± SEMs of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. Two-tailed Student's *t*-test (A, E, G, H, J) or one-way analysis of variance (ANOVA) followed by Bonferroni's test (F, I, K).

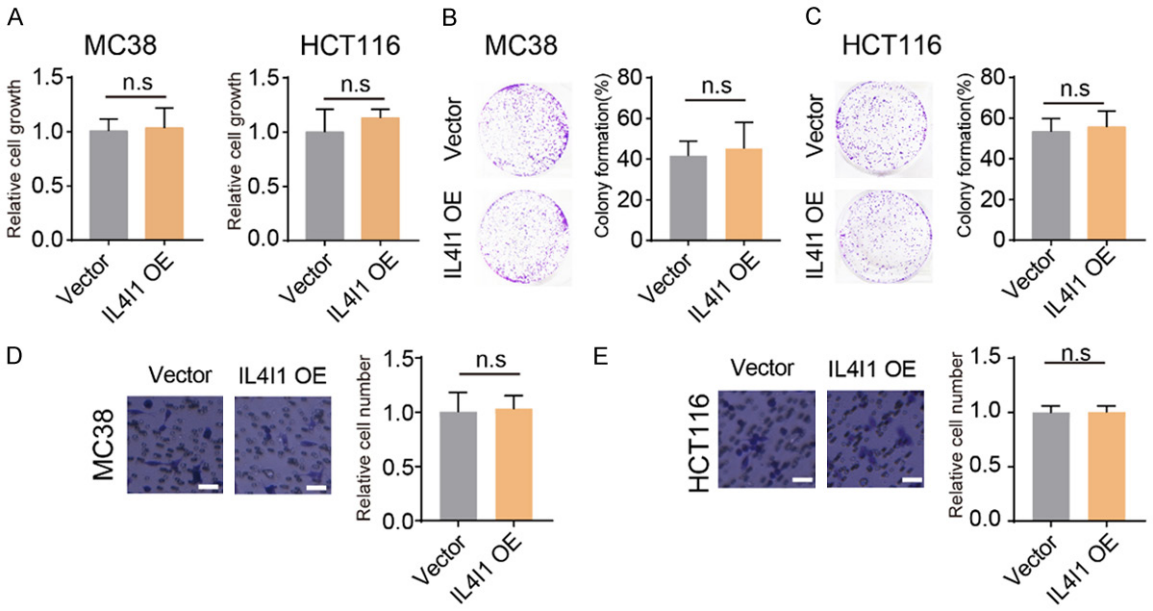


Figure 2. IL4I1 promoted the oncogenesis of colorectal cancer through tryptophan metabolism pathway. (A) MTT assay of the vector- and IL4I1-overexpressing MC38 cells and HCT116 cells cultured in Trp-free medium. (B, C) Colony formation analysis of the vector- and IL4I1-overexpressing MC38 cells (B) and HCT116 cells (C) cultured in Trp-free medium. (D, E) The relative number of the migrated vector- and IL4I1-overexpressing MC38 cells (D) and HCT116 cells (E) cultured in Trp-free medium. Bar = 40 μm. The data were presented as means ± SEMs of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. Two-tailed Student's t-test (A-E).

tion of *IL4I1* with *AHR* signature expression in colorectal cancer (**Figure 3A**). AHR is a ligand-activated cytoplasmic receptor and is translocated into nucleus to regulate gene expression upon activation. We therefore examined the cytoplasmic expression and the nuclear translocation of AHR by immunofluorescence staining and found that IL4I1 overexpression facilitated the nuclear translocation of AHR in MC38 and HCT116 cells (**Figure 3B**, **3C**). To further evaluate the above results, we conducted Nucleoplasmic separation experiments, and found that AHR increased in the nucleus after IL4I1 overexpression (**Figure 3D**). Moreover, similar results were obtained in patient tumor samples (**Figure 3E**), suggesting the potential role of AHR in IL4I1-induced tumor progression. Importantly, to directly demonstrate the functional importance of AHR in IL4I1 mediated oncogenesis, we used AHR inhibitor SR-1 to block AHR activity. The results showed that SR-1 treatment significantly attenuated the IL4I1 overexpression-induced proliferation, colony formation, and cell migration of MC38 and HCT116 cells (**Figure 3F-J**). Collectively, these data indicated the functional importance of AHR activation in IL4I1 promoted oncogenesis.

IL4I1 induced the adaptive immune dysfunction through an enhanced CD8+ T cell exhaustion

It has been well known that tumor progression is associated with numerous biological func-

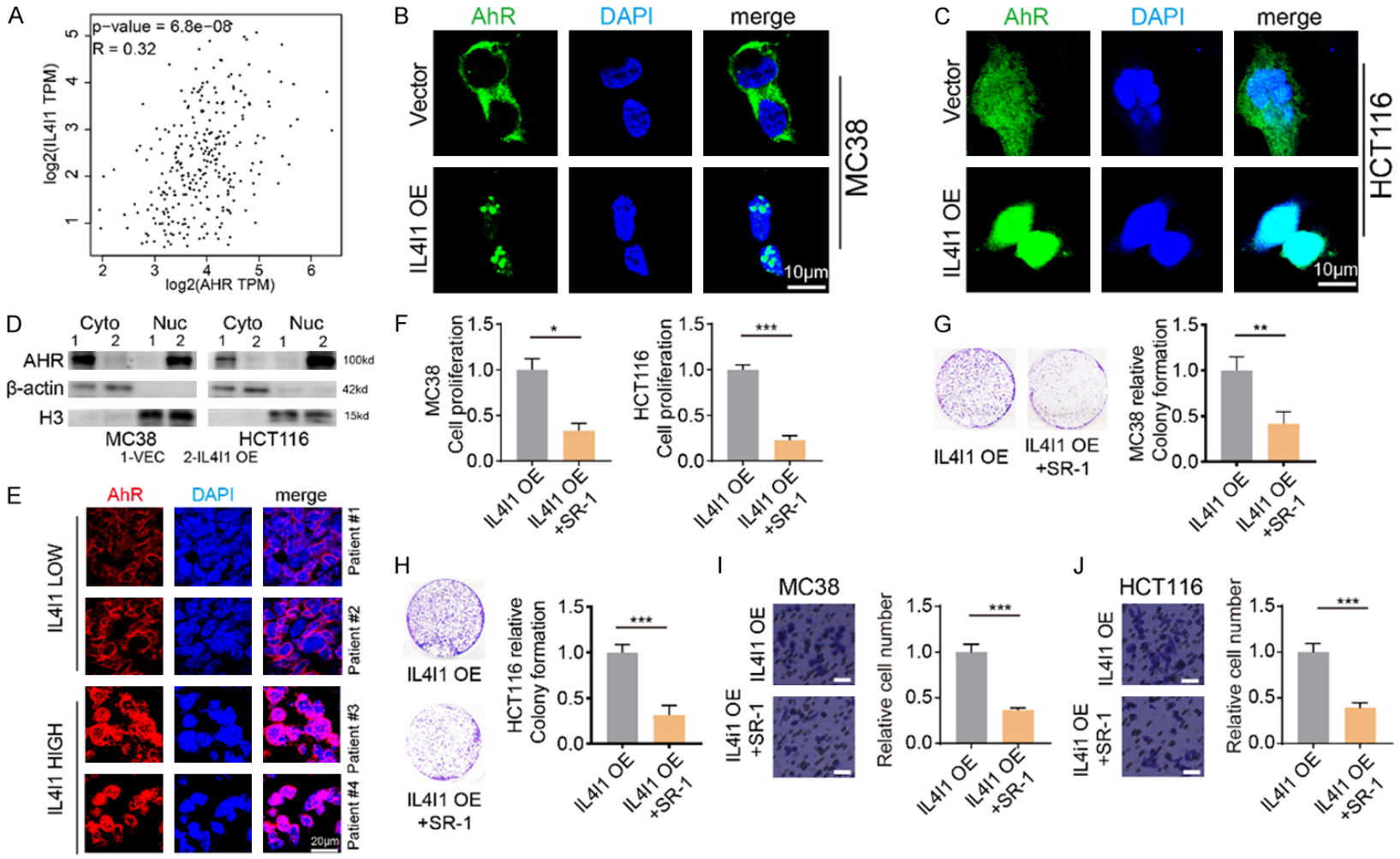


Figure 3. IL4I1 promoted oncogenesis through enhancing the nuclear translocation and the activation of AHR in colorectal cancer. (A) Correlation between the AHR and IL4I1 expression based on the TGGA cohort. (B, C) Immunofluorescence staining of AHR in vector- and IL4I1-overexpressing MC38 (B) and HCT116 cells (C). Bar = 10 μm. (D) Immunoblot of AHR in vector- and IL4I1-overexpressing MC38 and HCT116 cells. (E) Immunofluorescence staining of AHR in IL4I1-high or IL4I1-low patient samples. Bar = 20 μm. (F) MTT assay of IL4I1-overexpressing MC38 and HCT116 cells treated with DMSO or SR-1. (G, H) Colony formation assay of IL4I1-overexpressing MC38 (G) and HCT116 cells (H) treated with DMSO or SR-1. (I, J) The relative number of migrated IL4I1-overexpressing MC38 cells (I) and HCT116 cells (J) treated with DMSO or SR-1. Bar = 40 μm. The data were presented as means ± SEMs of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. Two-tailed Student's t-test (F-J) or Pearson's correlation test (A).

tions, including the activation of pro-survival signaling pathways in cancer cells and the immunosuppression in the tumor microenvironment [17]. Therefore, in this study, we investigated whether adaptive immune dysfunction, such as T cell dysfunction and exhaustion, was related to IL4I1-promoted tumor progression. We first explored the association between IL4I1 and T cell exhaustion signature expression by using TCGA database and found a positive correlation of IL4I1 with T cell exhaustion signature in colorectal cancer patients (**Figure 4A**). Then, we established patient tumor cell-derived tumor organoids to study the effect of tumor microenvironment on IL4I1 function. The tumor cells were infected with lentivirus to either overexpressing IL4I1 or depleting IL4I1. Overexpression of IL4I1 significantly prolonged tumor organoids growth in Matrigel, while IL4I1 knockout suppressed the cells proliferation and tumor organoids development (**Figure 4B**). Furthermore, we co-cultured the tumor-reactive CD8+ T cells (CTLs) with either IL4I1-overexpressing or IL4I1-depletion tumor organoids, and the results showed that the expression of exhaustion-associated surface markers, PD-1 and TIM3, was remarkably upregulated on CTLs co-cultured with IL4I1-overexpressing organoids compare with the vector-expressing group (**Figure 4C**). In addition, the expression of IFN-γ and TNF-α was lower in CTLs when co-cultured with IL4I1-overexpressing organoids compare than in vector-expressing control group (**Figure 4C**). Moreover, we determined the cytotoxic activity of CTLS after co-culturing with organoids harboring either IL4I1 overexpression or depletion for 12 h. CTLs showed weaker cytotoxic activity against IL4I1-overexpressing organoids than against vector-expressing group (**Figure 4D**). Conversely, CTLs showed strong cytotoxic activity against IL4I1-depletion organoids (**Figure 4E**).

AHR activation by IL4I1 was required for CD8+ T cell exhaustion

Since we have determined that AHR activation is important in IL4I1 promoted oncogenesis, we further explored if AHR activation had a role in T cell dysfunction. We co-cultured the tumor organoids with CTLs for 12 h and then examined the nuclear translocation of AHR in CTL cells. We found that IL4I1 overexpression in tumor organoids could promote AHR nuclear translocation in CTL cells (**Figure 5A**, **5B**). CTL cells with AHR knockdown by shRNA showed not only stronger cytotoxic activity against IL4I1-overexpressing tumor organoids (**Figure 5C**), but also lower exhaustion-associated phenotype than CTLs transfected with NC shRNA (**Figure 5D**, **5E**). Moreover, AHR inhibition by SR-1 combined with CTLs significantly increased apoptosis rate in tumor organoids (**Figure 5F**). Finally, using TCGA database, we found that patients with colorectal cancer expressing high level of IL4I1 exhibited worse overall survival than patients expressing low levels of IL4I1 (**Figure 5G**). Taken together, those results suggested that IL4I1/AHR might regulate colorectal cancer progression through modulating T cell activity.

Discussion

In the present study, we have explored the role of IL4I1 in the tumorigenesis, metastasis, and immune evasion in colorectal cancer. Our study revealed a higher expression of IL4I1 in tumor tissues, and the metabolites of Trp could activate AHR to promote the proliferation, migration, and immune evasion of colorectal cancer.

Previous studies have found that the AHR is constitutively overexpressed and activated and that AHR could serve as a therapeutic target [14, 18]. A recent study has shown that AHR inhibition sensitizes the human colorectal cancer models to EGFR tyrosine kinase inhibitor [19]. In this study, we treated the tumor organoids with AHR inhibitor SR-1 and found that AHR inhibition in combination with TCLs co-culture achieved superior activity than SR1 treatment or immunotherapy alone in colorectal cancer, supporting the notion of targeting AHR as a conjugate treatment of human colorectal cancer in clinic.

Numerous metabolites derived from Trp could activate AHR signaling and involve in biological processes during tumor progression. L-Kynurenine (Kyn) is a well-known tryptophan metabolite catalyzed by indoleamine 2,3-dioxygenase 1 and tryptophan 2,3-dioxygenase 3 (IDO/TDO) and interacts with AHR. Their interaction subsequently leads to an immunosuppressive microenvironment through inducing the Treg generation or the exhaustion phenotype of cytotoxic CD8+ T cells [20, 21]. Previous study has found a lower tryptophan level but higher

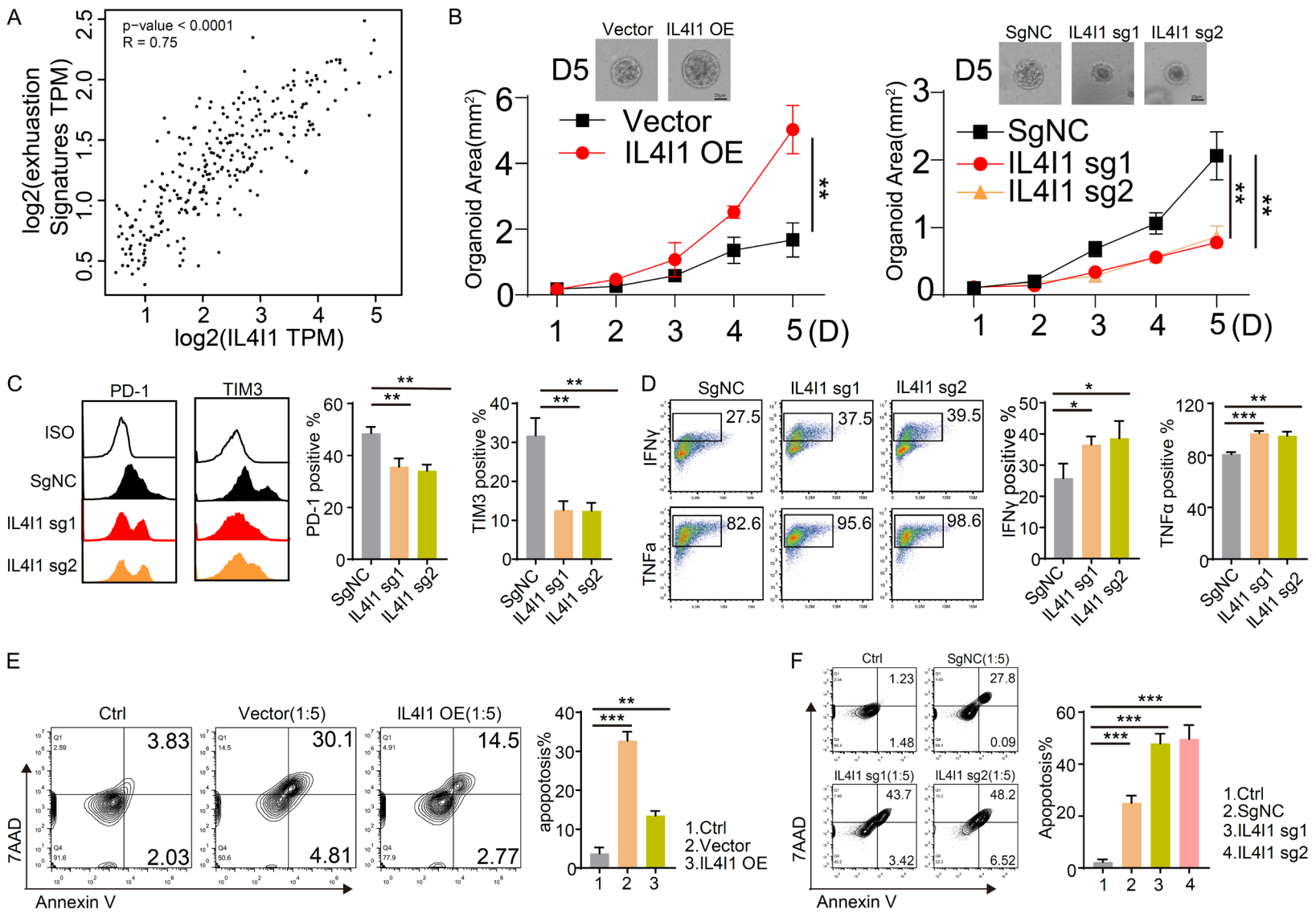
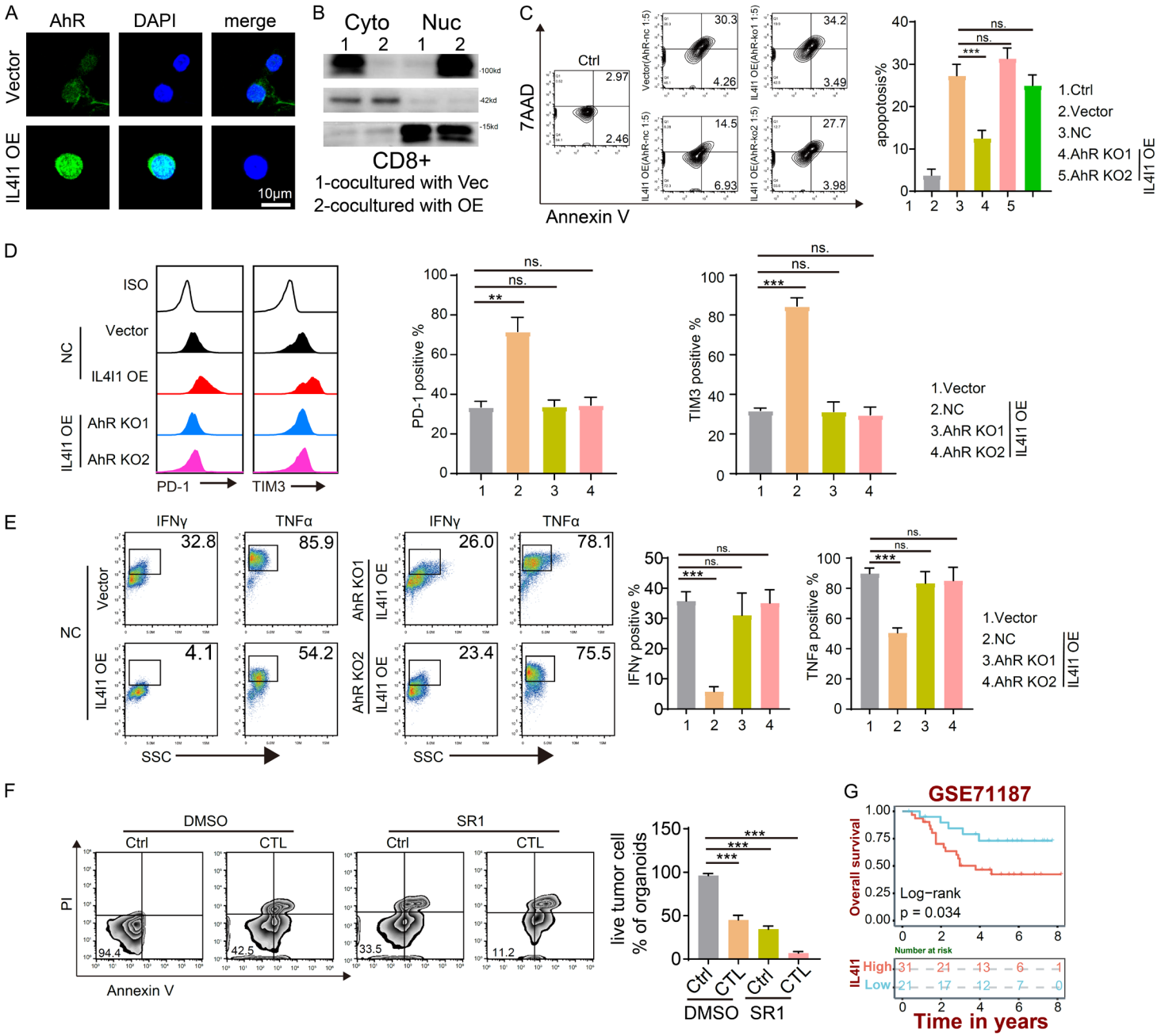


Figure 4. IL4I1 induced adaptive immune dysfunction through enhanced CD8+ T cell exhaustion in tumor microenvironments. (A) Correlation between the T cell exhaustion signature and IL4I1 expression based on the TGGA cohort. (B) The effect of IL4I1 knockout or overexpression on PDOs growth was evaluated. (C, D) The expression of PD-1, TIM3, IFNγ, and TNFα was analyzed by flow cytometry in CD8+ T cells co-cultured with PDOs that were either overexpressing IL4I1 or IL4I1 knockout. (E, F) Apoptosis analysis of PDOs that were either overexpressing IL4I1 or IL4I1 knockout and were treated with CTL for 24 h. Annexin V and 7AAD staining were performed and analyzed by flow cytometry. The data were presented as means ± SEMs of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. Two-tailed Student's t-test (B) or one-way analysis of variance (ANOVA) followed by Bonferroni's test (C-F) or Pearson's correlation test (A).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TNFα
32.8
85.9
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26.0
78.1
23.4
75.5
SSC
IFNγ positive %
TNFa positive %
F
DMSO
SR1
Ctrl
CTL
PI
Annexin V
94.4
42.5
33.5
11.2
live tumor cell
% of organoids
G
GSE71187
Overall survival
Log−rank
p = 0.034
Number at risk
IL4I1
High 31 21 13 6 1
Low 21 17 12 7 0
Time in years

Figure 5. AHR activation by IL4I1 was required for CD8+ T cell exhaustion. (A) CD8+ T cells that were co-cultured with PDOs were stained for AHR and DAPI; scale bar, 10 μm. (B) Immunoblot of AHR, H3, β-actin for CD8+ T cells that were co-cultured with PDOs. (C) After treatment with CTL-NC or CTL-shAHR for 12 h, PDOs cells were collected, stained with Annexin V and 7-AAD, and analyzed for cell apoptosis by flow cytometry. (D, E) CTL cells were transfected with either shNC or shAHR-expressing lentiviral particle and then were co-cultured with IL4I1-overexpressing PDOs. The expression of PD-1, TIM3, IFNγ, and TNFα was analyzed. (F) PDOs were treated with DMSO, SR-1, CTL or CTL combined with SR-1. After treatment for 24 h, PDOs cells were collected, stained with Annexin V and 7-AAD, and analyzed for cell apoptosis by flow cytometry. (G) The correction between patient overall survival and IL4I1 expression was analyzed in GEO71187 dataset. The data were presented as means ± SEMs of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. One-way analysis of variance (ANOVA) followed by Bonferroni's test (C-F). Or log-rank survival analysis (G).

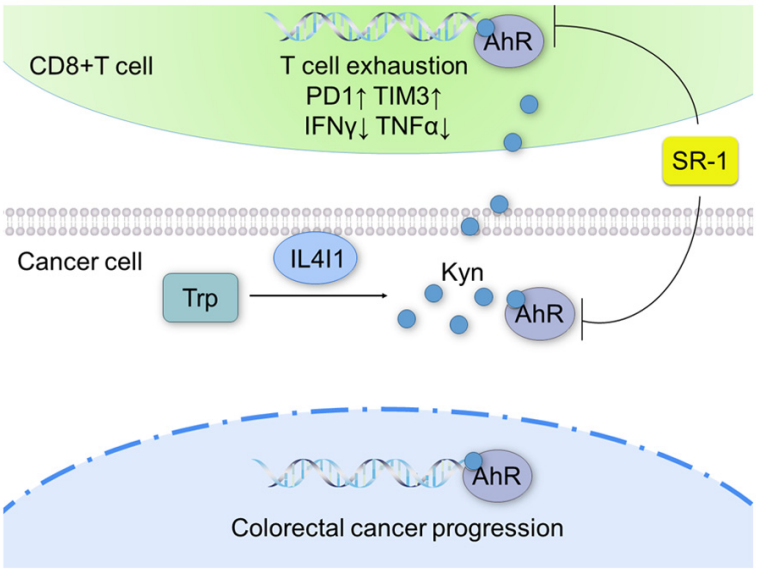

Figure 6. Graphical abstract.

tryptophan metabolism in the plasma of colorectal cancer, which is not associated with cancer outcome or response to chemotherapy [22-24]. In our present study, deprivation of Trp in cultural medium diminished IL4I1-induced tumor proliferation and migration, suggesting that the metabolites of Trp contributed to AHR activation, tumorigenesis, and invasion in colorectal cancer. In supporting this, we also found IL4I1 could rapidly drive T cell exhaustion via AHR signaling pathway.

IL4I1 is an L-amino acid oxidase that catalyzes phenylalanine into phenylpyruvic acid (PP) and H_2O_2. In addition to phenylalanine, IL4I1 can also catalyze tyrosine and tryptophan into hydroxyphenylpyruvc acid and indole-3-pyruvic acid (I3P) which is a ligand for the subsequent AHR activation [25, 26]. Several previous studies have demonstrated the immunosuppressive role of IL4I1 in various cancers [27, 28]. IL4I1 promotes Foxp3+ Tregs differentiation, inhibits the CD8+ antitumor T cell responses, and reduces Th1 and Th17 responses [29-31]. Recently, Sadik et.al were the first to link IL4I1 to tumor-intrinsic properties, including cancer cell growth and metastasis, through activating AHR by catabolizing Trp [14]. In addition to tumor intrinsic properties, Zeitler *et.al* found that the oncogenic effect of IL4I1 could be mediated by an anti-ferroptotic mechanism via generating indole-3-pyruvate (I3P) from Trp [32]. Our current study also linked IL4I1 to tumor intrinsic properties and demonstrated that IL4I1 could promote tumor proliferation, migration, and immunosuppression dependent on the presence of Trp. However, whether IL4I1 can shape the local tumor immune microenvironment or modulate tumor cell death pathways in colorectal cancer requires further investigation.

Conclusion

In conclusion, our present study found a higher IL4I1 expression in colorectal cancer tissues, and IL4I1 promoted tumorigenesis, metastasis, and the immunosuppression of tumor cells through tryptophan metabolism pathway. Further mechanistic study demonstrated that IL4I1 increased the nuclear translocation and the activation of AHR in colorectal cancer cells (**Figure 6**). Our findings suggested that drugs targeting IL4I1 might be a promising therapeutic approach for the treatment of colorectal cancer.

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CRC tissues and adjacent tissues were collected from The Fifth Medical Center of Chinese

PLA General Hospital. All the subjects signed the written informed consent form. This work was approved by the Medical Ethics Committee of The Fifth Medical Center of Chinese PLA General Hospital.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xue Ma, Department of Geriatric Medicine, The Sixth Medical Center of Chinese PLA General Hospital, Ganjiakou Street, Haidian District, Beijing 100000, China. E-mail: maxue728@163.com; Juyi Wen, Senior Department of Oncology, The Fifth Medical Center of Chinese PLA General Hospital, No. 100, West Fourth Ring Middle Road, Fengtai District, Beijing 100039, China. E-mail: wenjuyi@126.com

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