

•Research article•

***Panax notoginseng* saponins prevent colitis-associated colorectal cancer via inhibition IDO1 mediated immune regulation**

LI Xue-Ming^{1A}, YUAN Ding-Yi^{1A}, LIU Ya-Hui^{2A}, ZHU Lei¹, QIN Hong-Kun¹,
YANG Yu-Bing¹, LI Yan³, YAN Fang^{2*}, WANG Ya-Jing^{1*}

¹Department of physiology, School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, Nanjing 210009, China;

²Department of Pharmaceutical Analysis, School of pharmacology, China Pharmaceutical University, Nanjing 210009, China;

³Department of Chronic Communicable Disease, Jiangsu Provincial Center for Disease Prevention and Control, Nanjing 210009, China

Available online 20 Apr., 2022

[ABSTRACT] Colorectal cancer (CRC) is the third most lethal cancer and leading cause of cancer mortality worldwide. A key driver of CRC development is colon inflammatory responses especially in patients with inflammatory bowel disease (IBD). It has been proved that *Panax notoginseng* saponins (PNS) have anti-inflammatory, anti-oxidant and anti-tumor effects. The chemopreventive and immunomodulatory functions of PNS on colitis-associated colorectal cancer (CAC) have not been evaluated. This present study was designed to study the potential protective effects of PNS on AOM/DSS-induced CAC mice to explore the possible mechanism of PNS against CAC. Our study showed that PNS significantly alleviated colitis severity and prevented the occurrence of CAC. Functional assays revealed that PNS relieved immunosuppression of Treg cells in the CAC microenvironment by inhibiting the expression of IDO1 mediated directly by signal transducer and activator of transcription 1 (STAT1) rather than phosphorylated STAT1. Ultimately, Rh1, one of the PNS metabolites, exhibited the best inhibitory effect on IDO1 enzyme activity. Our study showed that PNS exerted significant chemopreventive function and immunomodulatory properties on CAC. It could reduce macrophages accumulation and Treg cells differentiation to reshape the immune microenvironment of CAC. These findings provided a promising approach for CAC intervention.

[KEY WORDS] *Panax notoginseng* saponins; Colitis-associated colorectal cancer; Chemoprevention; Treg cells; IDO1

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2022)04-0258-12

Introduction

Colitis-associated colorectal cancer (CAC) is a catastrophic complication of inflammatory bowel disease (IBD). IBD is more likely to develop into CAC due to continuous production of pro-inflammatory cytokines, DNA-damaging reactive oxygen species, and nitrogen species [1]. Clinical symptoms of CAC such as rectal bleeding or changes in bowel habits are attributed to underlying IBD. Meanwhile, it

is difficult to recognize early malignant lesions on colonoscopy [2]. Consequently, patients with CAC are frequently diagnosed with advanced-stage malignancies. However, CAC patients are currently treated with the same chemotherapy regimens as patients with sporadic colorectal cancer (CRC), while duration of response and overall survival are worse for CAC patients [3]. Therefore, it is urgent to attach importance to preventing from CAC.

Emerging evidences have revealed that CD4⁺ helper T cells, CD8⁺ cytotoxic T cells, regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs) and macrophages play critical roles in the progression of CAC [4-6]. Among these immune cells, Treg cells are engaged in sustaining immune homeostasis during pathological and physiological immune responses. Treg cells showed an activated phenotype in late stages of CAC by expressing programmed death receptor-1 (PD-1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), CD127 and TIM-3. These suggesting an increased suppressive capacity which promote immune escape and accelerate the development of CAC [7, 8]. Indoleamine 2, 3-di-

[Received on] 31-Dec.-2021

[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81872903 and 82173956), the "Double First-Class" University project of China Pharmaceutical University (CPU2018GY03) and Fundamental Research Funds for the Central Universities of China Pharmaceutical University (2016ZZD003).

[*Corresponding author] E-mail: cpuyanfang@hotmail.com (YAN Fang); cpuwjy@cpu.edu.cn (WANG Ya-Jing)

^AThese authors contributed equally to this work.

These authors have no conflict of interest to declare.

oxygenase 1 (IDO1) is a cytosolic enzyme involved in the rate-limiting step of the tryptophan (Trp) metabolism to kynurenine (Kyn) [9, 10]. It is well-documented that IDO1 expression in tumor cells is associated with tumor-infiltrating FoxP3⁺ Tregs while a negative association with CD8⁺ cytotoxic T cells in the primary tumors and metastatic tissues [11-16]. IDO1 has been confirmed to be regulated by STAT1 [17, 18], signal transducer and activator of transcription 3 (STAT3) [19], COX₂ [20], Bridging integrator 1 (BIN1) [21, 22] and nuclear factor-kappa B (NF-κB) [23].

PNS are the main active pharmaceutical ingredients extracted from the traditional Chinese herbal medicine *Panax notoginseng* (Burk) F. H. Chen (Sanqi). Accumulating studies have shown that PNS exert multiple pharmacological activities, such as anti-inflammation, anti-oxidation, inhibition of platelet aggregation, regulation of blood glucose and neuronal protection [24-27]. Notably, PNS or its main components alleviate colitis *via* inhibition of the phosphoinositide-3-kinase/protein kinase B (PI3K/AKT) and NF-κB signaling pathways [28, 29]. Moreover, previous reports have shown that PNS used alone or combined with conventional chemotherapy enhance anti-tumor efficacy in colorectal cancer cells [30-33]. However, the effect of PNS on chemoprevention and tumor immunity and its underlying mechanism remain poorly understood.

In this study, we identified the potential chemoprevention of PNS on CAC through suppressing STAT1/IDO1 which mediated Treg cells differentiation. The findings illustrate the underlying molecular mechanism of PNS involved in the chemopreventive and immunomodulatory effects and provide new therapeutic candidates for CAC.

Materials and Methods

Cell culture and isolation

Murine colon carcinoma CT26 cell line and human cervical cancer Hela cell line were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). CT26 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS). Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) with 10% FBS. Both cell lines were cultured under a humidified 5% (V/V) CO₂ atmosphere at 37 °C.

Spleen single-cell suspensions were isolated as described [34]. 18–20 g BALB/C mice were sacrificed for neck dislocation, soaked in 75% ethanol for 5 min, and a small incision was made on the left side of the abdomen, and the spleen was carefully removed. The spleen was placed in a petri dish containing 5–20 mL phosphate buffered saline (PBS). The single-cell suspension is made by cutting the organ into small pieces with scissors and then gently pressing it into a fine stainless steel wire mesh (70 μm) with a sterile syringe piston. Mouse peripheral blood mononuclear cells (PBMC) were isolated using mouse peripheral blood lymphocyte isolation Kit (P8620, Solarbio, China).

Colitis-associated colorectal cancer model

Pathogen-free C57BL/6 mice (male and female in half) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) at 6–8 weeks. They were exposed to a 12 : 12-hr light/dark cycle. The mice received an intraperitoneal injection of 10 mg·kg⁻¹ AOM (Sigma A5486, USA) and maintained with regular drinking water for 1 week. Then the mice received three cycles of DSS (MP Biomedicals, molecular weight 35–50 kDa) treatment, with each cycle including 2.5% DSS treatment for 1 week and regular water for 2 weeks [35]. Mice weight, diarrhea degree, and rectum bleeding degree were evaluated twice a week to evaluate disease activity index (DAI) [36]. Mice were euthanized at the end of the experiment with blood samples and colon tissues collected. All procedures were approved by the Committee on the Ethics of Animal Experiments of China Pharmaceutical University.

Plant materials

PNS were purchased from Dasf Biotech (batch number: DASF1423S75), the main contents of PNS were analyzed by HPLC method and the results were presented in Fig. S1. In addition to 3 weeks of DSS, mice were given 75 mg·kg⁻¹ PNS (low dose), 150 mg·kg⁻¹ PNS (high dose) or 75 mg·kg⁻¹ 5-ASA (Dibo Biology, Shanghai, K758001) dissolved in distilled water.

Acute peritonitis

75, 150 mg·kg⁻¹ PNS or 200 mg·kg⁻¹ Epacadostat (IN-CB024360, S7910, Selleck) were applied for 5 days in 8-week-old mice before the intraperitoneal injection of 700 μg Alum (dissolved in 0.2 mL sterile PBS). After 12 h, serum was collected and the peritoneal cavities were washed with 6 mL cold sterile PBS. CD11b⁺ Ly6G⁺ neutrophils present in the peritoneal exudate (PE) and CD3⁺ CD4⁺ helper T cells, CD3⁺ CD8⁺ cytotoxic T cells, CD4⁺ CD25⁺ FoxP3⁺ Treg cells present in PBMC were analyzed using flow cytometry. The concentration of IL-1β in the PE and IL-1β, IL-6, IL-4, IFN-γ in the serum were quantified by ELISA. Macrophage in PE were collected to detect IDO1 expression by Immunofluorescent.

Western blot

Cells were lysed in RIPA buffer (25 mmol·L⁻¹ Tris-HCl pH 7.6, 150 mmol·L⁻¹ NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a phosphatase inhibitor (FD1002, FUDE Biological Technology). Protein concentrations were quantified using BCA Protein Assay Kit (23228, Thermo Scientific). Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then incubated with antibodies against phosphorylated STAT1 (P-STAT1; Y701, D4A7, CST), STAT1 (66545-1-Ig, Proteintech), IDO1 (66528-1-Ig, Proteintech), β-actin (60008-1-Ig, Proteintech), followed by incubation with the appropriate secondary HRP-conjugated antibodies, and development with High-sig ECL Western Blotting Peroxide Buffer (180-5001W, Tanon) and High-sig ECL Western Blotting Luminol/Enhancer Solution (180-

5001B, Tanon).

Immunohistochemistry

Immunohistochemistry was performed using Streptavidin-biotin method detection system from ZSGB-BIO (SP9001). Sections were deparaffinized and rehydrated through a descending alcohol series, followed by antigens retrieval, and endogenous peroxidase activity blocking. The sections were then incubated with primary antibodies against FoxP3 (22228-1-AP, Proteintech), IDO1, IL-1 β (D3U3E, CST), PCNA (10205-2-AP, Proteintech) overnight at 4 °C followed by visualized with a two-step process and a DAB staining kit (ZSGB-BIO, China). Finally, slides were counterstained with hematoxylin, dehydrated, and mounted.

Immunofluorescent

For immunofluorescent analysis, Hela cells were seeded at 15 mm glass-bottom cell culture dishes (752001, Nest) and rested overnight to allow proper attachment followed by application of PNS for 24 h. To promote IDO1 expression, Hela cells were then stimulated with IFN- γ (20 ng·mL⁻¹) for another 24 h. The cells were washed twice with sterile PBS and then fixed in absolute methanol for 10 min at -20 °C. Followed by three washes with PBS, Hela cells were blocked in 5% bovine serum albumin (BSA) dissolved in PBS for 60 min. Primary antibodies against P-STAT1 and STAT1 were incubated at 4 °C overnight. Hela cells were washed three times with PBS followed by incubation with Alexa-488 or Alexa-647 (ab150077 or ab150155, Abcam) secondary antibodies for 60 min at room temperature. DAPI (C1002, Beyotime) was used for nuclear staining. Samples were imaged through Zeiss LSM700 co-focus microscope and analyzed by Zen software.

Flow cytometry

Single-cell suspensions of colon tissues were isolated following established procedures [37]. Briefly, colons of the mice were washed with EDTA to remove epithelium cells and digested with collagenase IV (V900893, Sigma) to liberate cell populations. Tissue digests were separated by centrifugation on a 40%/80% Percoll (Sigma) gradient. Cells at the 40%/80% interface were collected as the leucocytes enriched in lamina propria (LP). Harvested cells were stained with combinations of the following antibodies against CD3-APC (17-0032-82, eBioscience), CD4-FITC (11-0041-85, eBioscience), CD8-PE (12-0081-82, eBioscience), CD25-APC (17-0251-81, eBioscience), FoxP3-PE (126404, Biolegend), CD11b-APC (12-0112-82, eBioscience), F4/80-PE (11-4801-82, eBioscience), Ly6G-FITC (11-5931-81, eBioscience). For staining FoxP3, cells were stained with surface markers before fixation and permeabilization using the FoxP3 staining buffer kit (Thermo Scientific) according to manufacturer instructions. Cell identification was performed using MACS Quant Flow Cytometer (Miltenyi). Data were analyzed using FlowJo (Tree Star, USA).

RNA extraction, cDNA synthesis and qPCR

RNA was isolated using RNA-easy Isolation Reagent (R701, Vazyme) followed by reverse transcription using HiS-

cript II 1st Strand cDNA Synthesis Kit (R211, Vazyme). qPCR was performed using Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) (11202ES03, YEASEN).

Splenocytes and CT26 cells co-culture model

CT26 cells were treated with mitomycin C (MMC, M5353, Sigma) at 37 °C for 30 min and placed on a 96-well plate according to 10⁴ cells per well after washing three times. Splenocytes of BALB/C mice were extracted on the next day and co-cultured with CT26 cells on the 96-well plate for 3 days, keeping the number of splenocytes was three times that of CT26 cells per well. Then Cell Counting Kits (CCK8, 40203ES60, YEASEN) were utilized to detect the proliferation of splenocytes. To analyze the effect of PNS on Treg cells, splenocytes were gained and stained with CD4-FITC, CD25-APC, FoxP3-PE to determine the proportion of Treg cells using flow cytometry.

IDO activity assay

Since kynurenine is the product of IDO1-dependent catabolism of tryptophan, the biological activity of IDO1 was evaluated by monitoring the level of kynurenine in the culture medium. 100 μ L of culture supernatant was mixed with 25 μ L of 30% trichloroacetic acid (TCA) diluted with water, vortexed and incubated in a 50 °C shaker for 30 min. Then centrifuged at 10 000 $\times$ g for 10 min. After that, 100 μ L supernatant was added to the equal volume of P-Dimethylbenzaldehyde (PDAB, 2% diluted with glacial acetic acid) in a 96-well plate and incubated at room temperature for 10 min. The absorbance at 492 nm was determined. The concentration of kynurenine was quantified using a standard curve generated from defined kynurenine concentrations (0–500 μ mol·L⁻¹).

Statistics

Each experiment was repeated at least three times to ensure the reliability of the results. All data were represented by mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM). Significant differences between the groups were estimated by Unpaired *t* test or one-way ANOVA. A value of *P* < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad 8.0 (GraphPad Software, USA).

Result

PNS alleviated AOM/DSS-induced colitis and suppressed the growth of colorectal adenomas.

Firstly, we established the CAC model that combined AOM-induced carcinogenesis and DSS-induced chronic inflammation as followed in Fig. 1A. Our results demonstrated that PNS significantly alleviated colitis symptom through preventing body weight loss, colon shortness and decreasing the DAI scores compared to AOM/DSS group (Fig. 1B–E). Moreover, we also confirmed that PNS suppressed tumor growth with the reduction of tumor number, polys size and tumor load in a dose-dependent manner. In addition, high dose of PNS was prior to the positive drug 5-ASA in tumor suppression (Fig. 1F–H). Meanwhile, PNS treatment significantly attenuated inflammatory cells infiltration in the mucosa

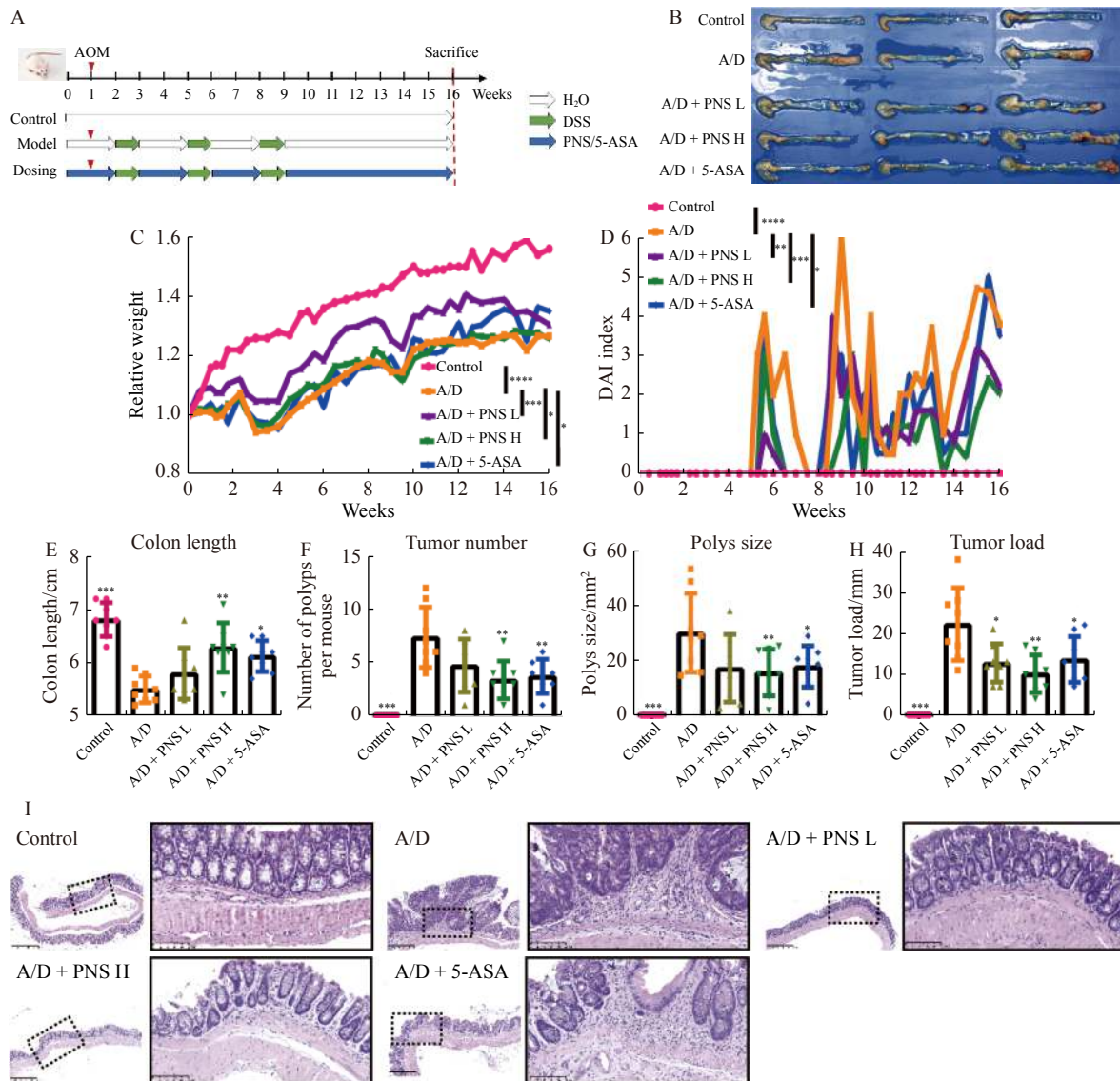


Fig. 1 PNS alleviated AOM/DSS-induced colitis and suppressed the growth of colorectal adenomas. (A) The treatment scheme designed for the AOM/DSS model of CAC. (B) Representative images of colon tissues. (C) Relative weight changes along with the whole process. (D) Average DAI changes along with the whole process. Clinical parameters include weight loss, stool consistency and bleeding of indicated mice. (E) Colon length. (F) Tumor number. (G) Tumor polyps size. (H) Tumor load (total diameters of the tumors on the colon tissue of each mouse). (I) H&E staining (scale bar = 200 μ m). Values are mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 vs AOM/DSS group by one-way ANOVA

and maintained integrity of the colonic epithelium, thus leading to the protective effect on the colons (Fig. 1I). Taken together, two doses of PNS significantly relieved AOM/DSS-induced colon inflammation and tumorigenesis.

To further explore the immunomodulatory effect of PNS, we established acute peritonitis (AP) model regarding INCB as the positive drug to verify the immunoregulatory effect of PNS. The results showed PNS inhibited the aggregation of CD11b⁺ Ly6G⁺ neutrophils in peritoneal exudate (Fig. S2A). Moreover, PNS alleviated the concentration of IL-1 β in peritoneal exudate while there was no difference in cytokine production in the serum such as IL-1 β , IL-6, IL-4 and IFN- γ (Fig. S2B). We supposed that anti-inflammatory effect of

PNS might be concentrated in the primary tissue and there was no enough time to affect the blood in condition of acute inflammation. Consistent with activation of adaptive immune responses, we observed an increase in the CD8 : CD4 T cell ratio and a downtrend of Treg proportion treated with PNS (Fig. S2C and S2D). Besides, we also found that PNS suppressed the expression of IDO1 in peritoneal macrophages (Fig. S2E).

PNS attenuated macrophage accumulation and Treg cells proportion in the CAC model

To clarify the mechanism underlying the effect of PNS on colon inflammation, the percentage of macrophages in colon tissues was analyzed and the results showed that high

dose of PNS reduced the aggregation of macrophages, which might explain the consequence of colon length and DAI index influenced by PNS (Fig. 2A and S3A). Furthermore, pro-inflammatory cytokine IL-1 β in the serum was significantly reduced by high or low dose of PNS which was better than 5-ASA. However, IL-4 and IL-6 in the serum exhibited no significant changes among the groups (Fig. 2B). Macrophage is one of the most dominant sources of IL-1 β , so it was reason-

able to assume low IL-1 β concentration in the colon due to macrophage reduction. To prove the hypothesis, we evaluated the expression of IL-1 β in colon tissues by IHC and found the expression of IL-1 β was indeed suppressed by PNS in the colonic epithelium and lamina propria (Fig. 2C).

Spleen index is the mass ratio of spleen to body weight of mice, PNS effectively alleviated splenomegaly caused by AOM/DSS, reflecting an immunoregulatory effect of PNS

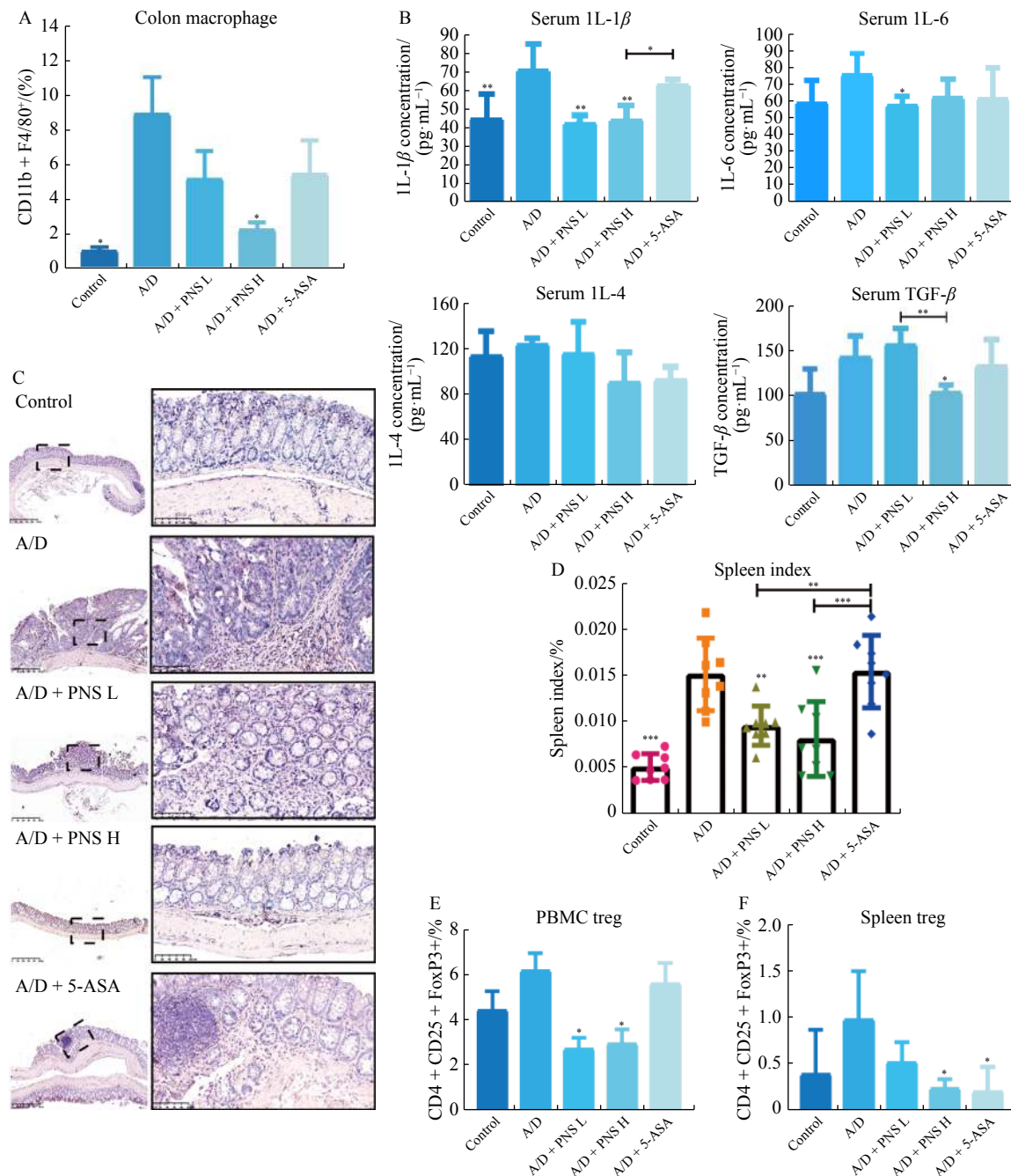


Fig. 2 PNS attenuated macrophage accumulation and Treg cells proportion in the CAC model. (A) Percentage of CD11b⁺ F4/80⁺ macrophages in colon lamina propria (LP) cells. (B) The concentration of IL-1 β , IL-6, IL-4 and TGF- β in the serum. (C) The protein expression of IL-1 β in colon tissues for IHC analysis (scar bar = 20 μ m). (D) Spleen index. (E) Percentage of CD4⁺ CD25⁺ FoxP3⁺ Treg cells in PBMC. (F) Percentage of CD4⁺ CD25⁺ FoxP3⁺ Treg cells in spleens. Values are mean $\pm$ SEM (n = 3). * P < 0.05, ** P < 0.01, *** P < 0.001 vs A/D group by Unpaired t test

(Fig. 2D). To identify the mechanism of PNS on tumor suppression, we isolated lymphocytes from peripheral blood, spleen and colon tissues for flow cytometry analysis. Our result revealed that PNS ameliorated the percentage of Treg in PBMC and splenic lymphocytes (Fig. 2E–F and S4A–S4B) while PNS showed no significant influence on CD8 : CD4 T cells ratio in PBMC or CD3⁺ total T cells in colon tissue (Fig. S3B and S3C). In summary, the immunoregulatory effect of PNS on tumor suppression mainly focused on Treg cells.

PNS alleviated T cell differentiation into Treg cells through IDO1.

In order to confirm the effect of PNS on Treg proportion, we analyzed the expression of FoxP3 expression through

IHC. The resulted reflected the distribution of Treg cells was alleviated by PNS in colon lamina propria (Fig. 3A). CD4⁺ naive T cells could differentiate into Treg cells when stimulated by some immuno- suppressive factors such as TGF- β released by tumor cells [38]. So we utilized CT-26 cells co-cultured with freshly isolated splenocytes to establish an *in vitro* model to further investigate the effect of PNS on the proliferation and differentiation of Treg cells, both of them revealed that PNS suppressed the differentiation of Treg cells and promoted the proliferation of splenocytes consistent with the results *in vivo* (Fig. 3B–C). Furthermore, we checked related immune checkpoints (ICKs) and found that the mRNA levels of CTLA-4 and LAG-3 in splenocytes of the co-culture model were not influenced by PNS. Although PNS downregulated

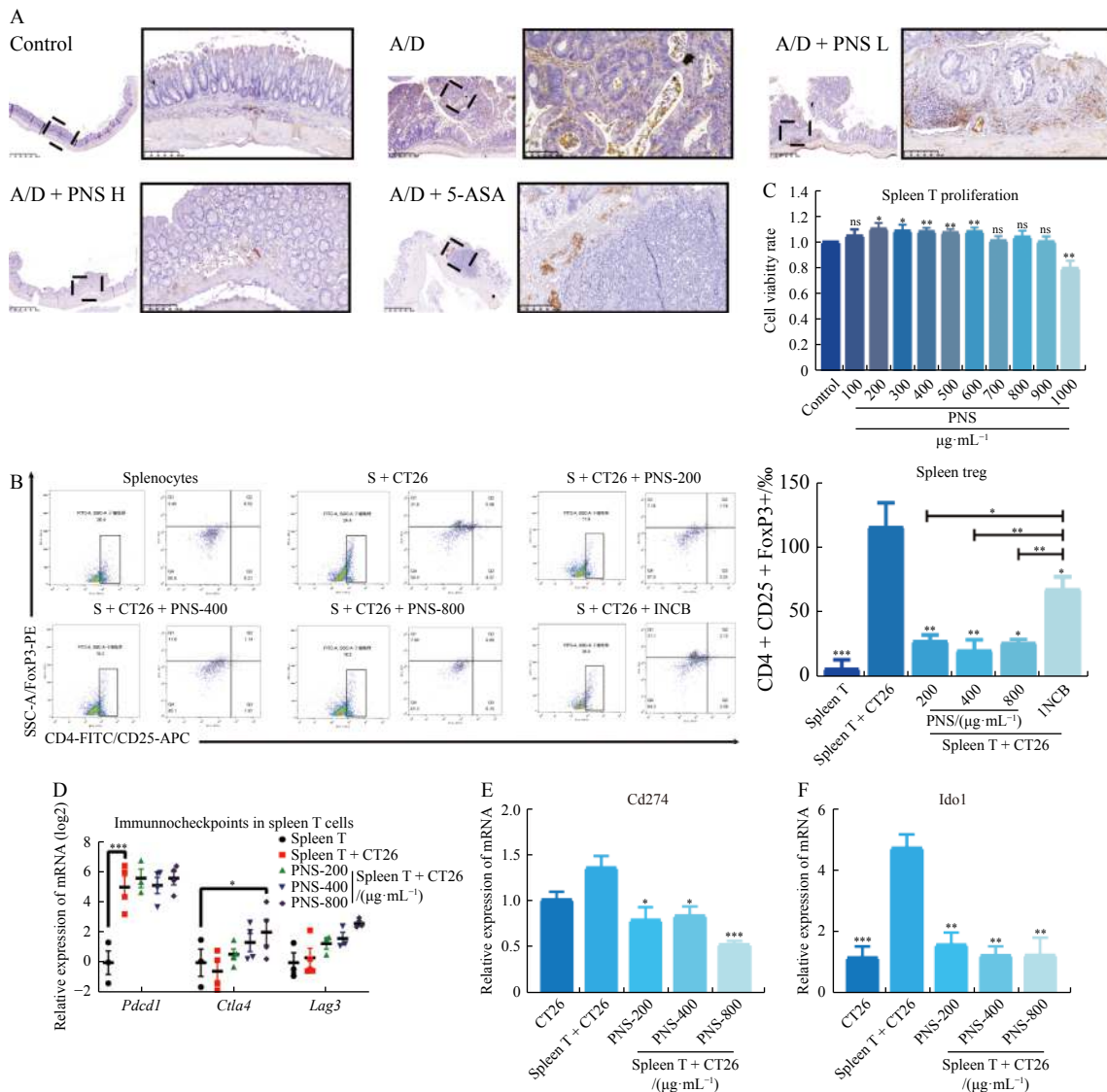


Fig. 3 PNS alleviated T cell differentiation into Treg through IDO1. (A) IHC for FoxP3 protein expression in colon tissues (scar bar = 20, 200 μ m). (B) The proportion of Treg cells in CT26-splenocytes co-culture model ($n = 3$, * $P < 0.05$, ** $P < 0.01$ vs T + CT26 group). (C) The proliferation of splenocytes (abbreviated as S) in CT26-splenocytes co-culture model ($n = 4$, values are mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, ns means not significant vs con group). (D) The gene expressions of PD-1, CTLA-4, LAG-3 in splenocytes of the co-culture model. (E, F) The gene expressions of PD-L1 and IDO1 in CT26 cells of the co-culture model ($n = 3$, values are mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs Co-CT26 group). Statistics were analyzed by Unpaired t test

the transcription level of PD-L1 in CT26 cells, it exerted no effect to PD-1 expression in splenocytes. Notably, PNS significantly suppressed IDO1 expression on the transcription level among these ICKs (Fig. 3D–3F). Combined with the above about IDO1 inhibition in the acute peritonitis (AP) model, PNS might exert immunoregulatory effect on Treg cells through modulating the expression of IDO1.

PNS suppressed IDO1 expression through modulating STAT1 expression.

In order to verify whether PNS indeed play the roles in Treg differentiation by regulating IDO1, we analyzed the expression of IDO1 in colon tissues in the CAC model. According to our immunohistochemical data, PNS effectively inhibited the expression of IDO1 in colon tissues of AOM/DSS-exposed mice (Fig. 4A). To further explore the mechanism of PNS on IDO1, we established an *in vitro* model stimulated by recombinant IFN- γ (20 ng·mL⁻¹) to induce endogenous IDO1 expression in Hela cells. 800 μ g·mL⁻¹ PNS significantly decreased the mRNA abundance and protein expression of IDO1 (Fig. 4B and 4D). Genes downstream IFN- γ signaling that might affect IDO1 expression were analyzed by qPCR (Fig. 4C and S4C). Similar to the result of IDO1, 800 μ g·mL⁻¹ PNS suppressed the transcription and protein expression of STAT1 (Fig. 4C and 4D). Furthermore, immunofluorescent analysis revealed the stable suppression of STAT1 and P-STAT1 by PNS (Fig. 4E and S4E). Except for STAT1, PNS alleviated STAT3 transcription *in vitro* model while showed no significance on P-STAT3 expression in colon tissues in AOM/DSS model implying that IDO1 suppression by PNS might not be through STAT3 (Fig. S4C and S4D).

PNS primarily targeted STAT1 rather than P-STAT1.

It has been well investigated that the homologous dimer of P-STAT1 translocates into the nucleus and binds to the IDO1 promoter to activate IDO1 expression^[39]. The phosphorylation process can be completed in a short time. In order to confirm whether PNS directly acted on P-STAT1, we evaluated the expression of STAT1 and P-STAT1 after applying IFN- γ for 1, 3, 12 h. The results demonstrated that PNS had no effect on the expression of P-STAT1 and IDO1 expressions in 1 and 3 h until the expression of STAT1 was decreased by PNS in 12 h implying that PNS modulated P-STAT1 and IDO1 through STAT1 expression (Fig. 5A and S5A). Moreover, we utilized JAK1/2 inhibitor Baricitinib to decrease the phosphorylation of STAT1. After pretreatment with Baricitinib, the expression of P-STAT1 and IDO1 upon IFN- γ treatment could be downregulated significantly while the expression of STAT1 mostly influenced by PNS rather than the inhibitor. Combined with Baricitinib, 800 μ g·mL⁻¹ PNS still reduced the expression of IDO1 reflecting that PNS mainly affects IDO1 protein expression by directly affecting STAT1 total protein expression (Fig. 5B and S5B). What's more, the reduction of STAT1 and P-STAT1 in colon tissues of CAC model confirmed STAT1 function on the immunomodulatory effect of PNS (Fig. 5C and 5D).

PNS metabolite Rh1 significantly inhibited the enzyme activity of IDO1

It is necessary to study the efficacy of monomers in PNS for better clinical applications. Rg1, Rb1, R1 are the main components of PNS and its contents in PNS are showed in Fig. S4. The analysis illustrated that the metabolite Rh1 can inhibit IDO1 enzyme activity at a very low concentration (2.5 μ g·mL⁻¹) while only after reaching a higher concentration (400, 200 μ g·mL⁻¹ respectively) did Rb1, R1 inhibit IDO1 enzyme activity (Fig. 6). Therefore, Rh1 might be regarded as a potential monomer of PNS to prevent CAC.

Discussion

Until now, combination chemotherapy regimen of irinotecan, fluorouracil, and oxaliplatin (OXP) has been regarded as the first-line treatment for advanced CAC after surgery^[40]. But low selectivity, insufficient concentrations in tumor tissues, and systemic toxicity in most patients limit the anti-tumor effect of the combined chemotherapy^[41,42]. So it is urgent to improve the efficacy of existing treatments or explore new approaches to protect from CAC. Many researches indicated that Chinese herbal injection had obvious advantages in reducing toxicity of chemotherapy, enhancing life quality and improving short- and long-term efficacy^[43]. As one of the most common Chinese herbal medicine, notoginseng significantly enhanced the anti-proliferation effect of 5-FU suggesting a possible reduction in the dose of 5-FU when used in combination with notoginseng thereby decreasing the drug's dose-related side effects^[33]. Another study showed that notoginseng enhanced the cytotoxicity of cisplatin by increasing the gap junction's intercellular communication^[28]. Therefore, PNS might be regarded as an adjuvant combined with chemo-therapies for better clinical treatment. Importantly, we identified the chemopreventive effect of PNS on CAC so that PNS might be applied for decreasing the occurrence of CAC as an intervention treatment.

Recently, various ICK inhibitors targeting PD-1/PD-L1 and CTLA-4 have been developed to benefit patients with deficient mismatch repair (dMMR)/high levels of microsatellite instability (MSI-H)^[44]. We confirmed the immunoregulatory effect of PNS on CAC is based on STAT1-dependent expression of IDO1 to suppress Treg cells differentiation for the first time. Moreover, except for IDO1, PNS suppressed the gene expression of PD-L1 in CT26 cells in the co-culture model reflective of PNS effect on immunotherapy. Takeshi Iwasaki et al found that the expression of PD-L1 and IDO1 positively correlated with IFN- γ and STAT1 expression in soft tissue leiomyosarcoma (LMS)^[45] which suggests changes in the expression of IDO1 and PD-L1 in the co-culture model in our study might be similarly influenced by STAT1. Notably, the mechanism between STAT1 and IDO1 we illustrated was limited in IFN- γ activating pathways which mainly included JAK/STAT1 and NF- κ B signaling pathways. Here, PNS could not influence NF- κ B mRNA expression illustrating that the possibility of NF- κ B pathway was ex-

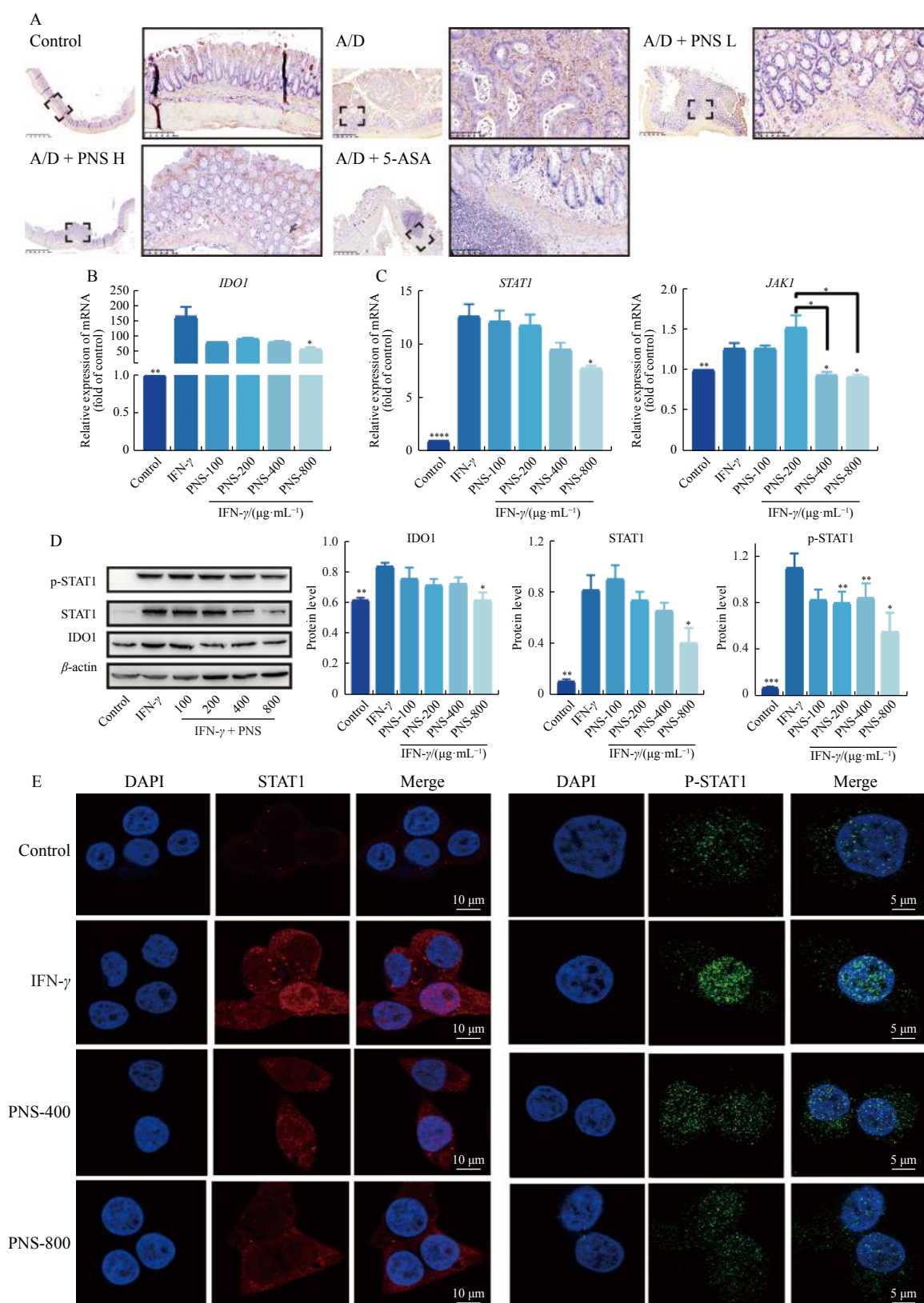


Fig. 4 PNS suppressed IDO1 expression through modulating STAT1 expression. (A) IHC analysis of IDO1 expression in colon tissues (scar bar = 20, 200 μm). (B, C) The gene expression of IDO1, STAT1 and JAK1 in HeLa cells stimulated by IFN- γ . (D) The protein expression of STAT1, P-STAT1 and IDO1 in HeLa cells stimulated by IFN- γ . (E) IF analysis of STAT1 (Red) and P-STAT1 (Green) expression in HeLa cells stimulated by IFN- γ (1260 $\times$). $n = 3$, values are mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs IFN- γ group, statistics were analyzed by Unpaired t test

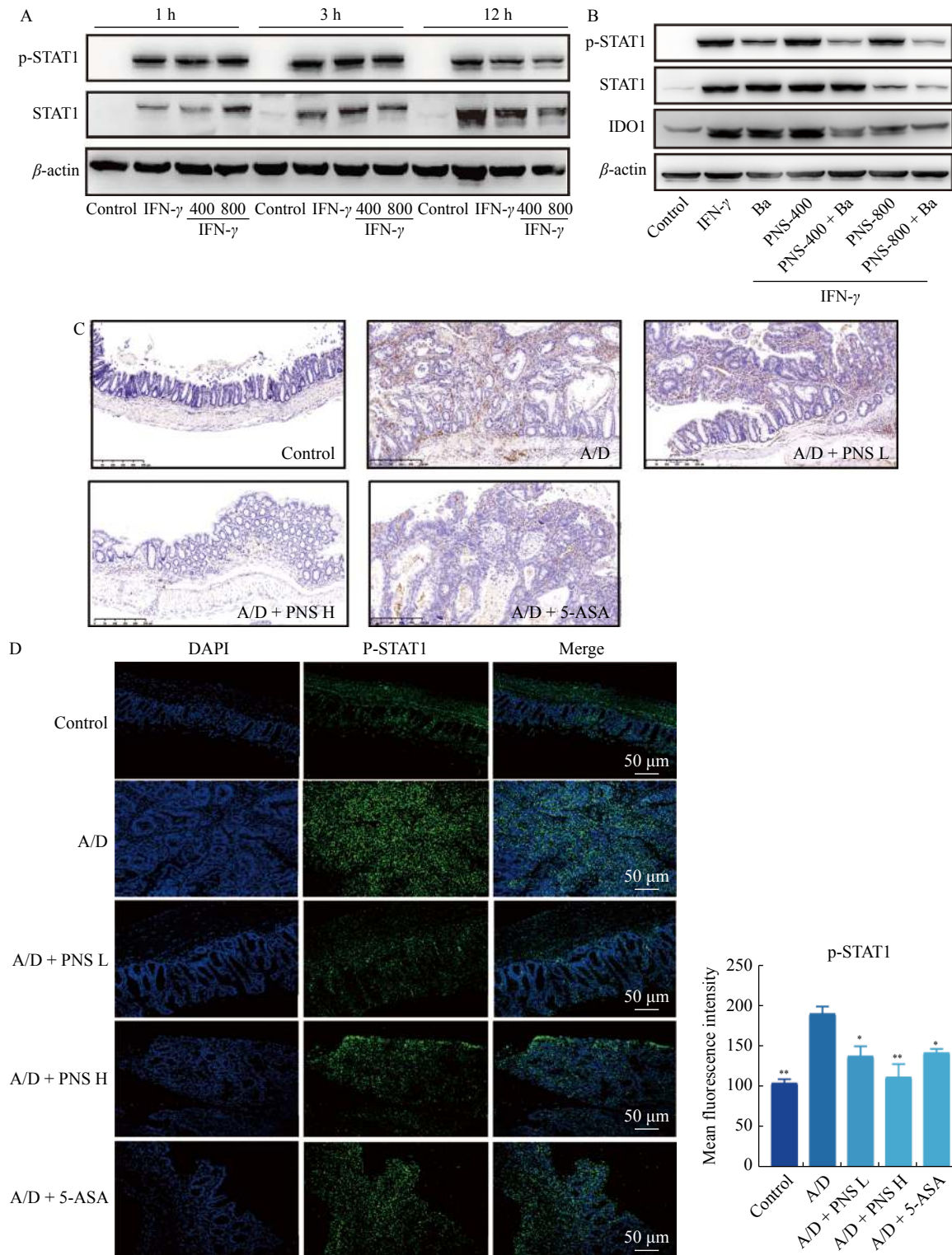


Fig. 5 PNS primarily targeted STAT1 rather than p-STAT1. (A) The protein expressions of p-STAT1 and STAT1 in HeLa cells for 1, 3, 12 h. (B) The protein expressions of p-STAT1, STAT1 and IDO1 in HeLa cells after applying Baricitinib (Ba, JAK1/2 inhibitor). (C) IHC analysis of STAT1 colon tissues in the CAC model in HeLa cells (scar bar = 20, 200 μm). (D) IF analysis of p-STAT1 in colon tissues in the CAC model (200 ×). Values are mean ± SEM (*n* = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs A/D group by Unpaired *t* test

cluded and the inhibitory effect of PNS on IDO1 expression was indeed through modulating STAT1.

The immunometabolic IDO1 pathway has emerged as an important therapeutic target for solid tumors including

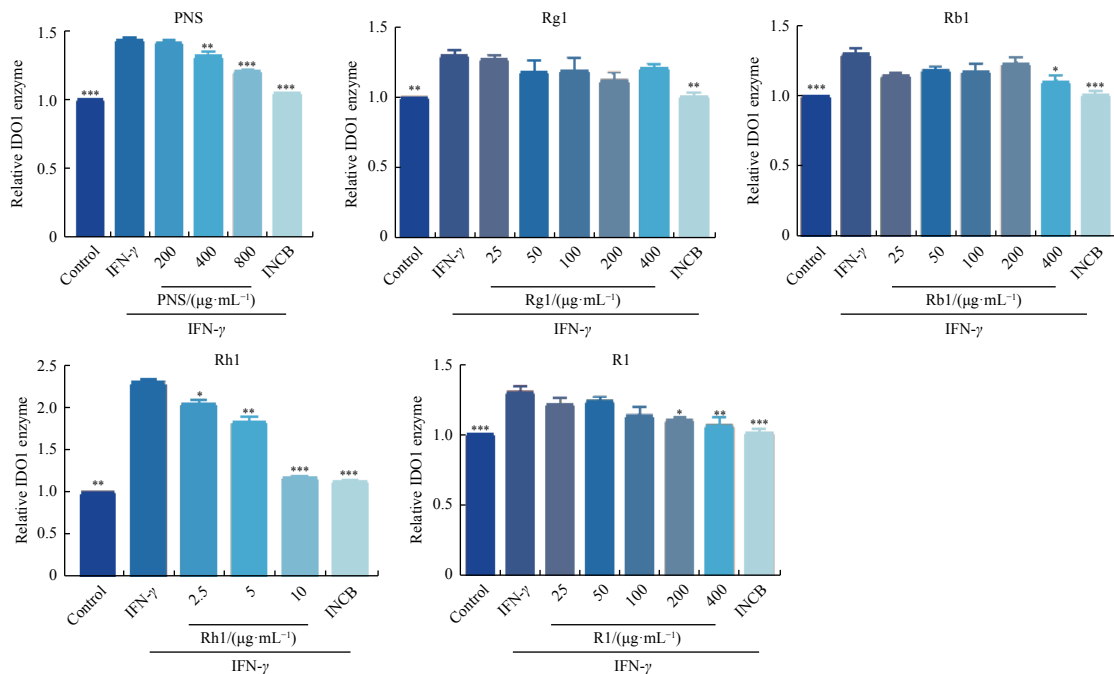


Fig. 6 PNS metabolite Rh1 significantly inhibited the enzyme activity of IDO1. The influence of PNS, Rg1, Rb1, Rh1, R1 on the enzyme activity of IDO1 ($n = 3$, mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, statistics were analyzed by One-way ANOVA)

colorectal cancer [46]. Notably, except for T cell immunity, IDO1 is inseparable from inflammation. Under normal physiological conditions, IDO1 plays a homeostatic role in regulating mucosal immunity in response to the local intestinal microbiota stimulus [47]. Associated research displayed that impairing de novo NAD⁺ synthesis through IDO1 inhibition enhanced proinflammatory responses in macrophages [48]. Therefore, active inflammatory responses caused by IDO1 inhibition is an inevitable question for Epacadostat or other ICK inhibitors targeted on IDO1. In our study, PNS not only inhibited the enzyme activity and expression of IDO1 but also suppressed the secretion of IL-1 β , thus well counteracted the increased inflammatory response caused by IDO1 inhibition. Meanwhile, it has been proved that Rh1 suppressed inflammation stimulated by LPS through TAK1/STAT3 or TLR4/STAT1 pathways [49]. Combined with our conclusion that Rh1 significantly inhibited the enzyme activity of IDO1, Rh1 could be a potential minor ginsenoside to exert favorable efficacy in immunotherapy. To sum up, PNS might be potential candidates combined with IDO1 inhibitors for better effects in immunotherapy.

STAT1 exerts importance in the innate and adaptive immunity and protects from pathogen infections. Activated STAT1 modulates diverse cellular processes, such as proliferation, differentiation and cell death [50]. Consistent with our conclusion, Pflügler *et al.* found that Treg cells were significantly reduced in STAT1^{ΔIEC} Apc^{Min} tumors and tumor cell-intrinsic STAT1 suppressed stroma immune cell activation through IDO1. Moreover, they confirmed strong co-expression between STAT1 and IDO1 at the mRNA level derived

from TCGA data [51]. Interestingly, p-STAT1 regulates transcription of protein-encoding genes, including STAT1 itself [50]. Therefore, STAT1 inhibition by PNS reduced P-STAT1 which in turn further decreased STAT1 transcription. Moreover, it has been proved that forced STAT1 expression or IFN treatment increased the expression of other IFN signaling pathway components, such as STAT2, IRF9 and IRF1 and then these transcription factors bind to STAT1 promoter, forming a positive feedback control of STAT1 expression [52]. While the transcription factors related to the positive feedback were not mentioned in our study, we could only speculate that PNS might block the positive feedback of STAT1 leading to reduced STAT1 expression.

Our findings collectively highlighted that PNS played chemopreventive roles on CAC and functioned as an immunomodulator by downregulating Treg differentiation mediated by IDO1. To date, Chinese herbal therapies are still in initial stages. Our novel data establish the role of PNS as a potential intervention target in the initiation and development of CAC.

References

- [1] Lasry A, Zinger A, Ben-Neriah Y. Inflammatory networks underlying colorectal cancer [J]. *Nat Immunol*, 2016, 17(3): 230-240.
- [2] Kameyama H, Nagahashi M, Shimada Y, *et al.* Genomic characterization of colitis-associated colorectal cancer [J]. *World J Surg Oncol*, 2018, 16(1): 121.
- [3] Yaeger R, Paroder V, Bates DDB, *et al.* Systemic chemotherapy for metastatic colitis-associated cancer has a worse outcome than sporadic colorectal cancer: matched case cohort analysis [J]. *Clin Colorectal Cancer*, 2020, 19(4): e151-e156.

- [4] Fantini MC, Favale A, Onali S, et al. Tumor infiltrating regulatory T cells in sporadic and colitis-associated colorectal cancer: the red little riding hood and the wolf [J]. *Int J Mol Sci*, 2020, **21**(18): 6744.
- [5] Basile D, Garattini SK, Bonotto M, et al. Immunotherapy for colorectal cancer: where are we heading? [J]. *Expert Opin Biol Ther*, 2017, **17**(6): 709-721.
- [6] Jiao Q, Ren Y, Ariston Gabriele AN, et al. Advances of immune checkpoints in colorectal cancer treatment [J]. *Biomed Pharmacother*, 2020, **123**: 109745.
- [7] Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells [J]. *Immunity*, 2013, **38**(3): 414-423.
- [8] Olguin JE, Medina-Andrade I, Molina E, et al. Early and partial reduction in CD4⁺Foxp3⁺ regulatory T cells during colitis-associated colon cancer induces CD4⁺ and CD8⁺ T cell activation inhibiting tumorigenesis [J]. *J Cancer*, 2018, **9**(2): 239-249.
- [9] Gide TN, Allanson BM, Menzies AM, et al. Inter- and intrapatient heterogeneity of indoleamine 2, 3-dioxygenase expression in primary and metastatic melanoma cells and the tumour microenvironment [J]. *Histopathology*, 2019, **74**(6): 817-828.
- [10] Wang S, Wu J, Shen H et al. The prognostic value of IDO expression in solid tumors: a systematic review and meta-analysis [J]. *BMC Cancer*, 2020, **20**(1): 471.
- [11] Ino K, Yamamoto E, Shibata K, et al. Inverse correlation between tumoral indoleamine 2, 3-dioxygenase expression and tumor-infiltrating lymphocytes in endometrial cancer: its association with disease progression and survival [J]. *Clin Cancer Res*, 2008, **14**(8): 2310-2317.
- [12] Moretti S, Menicali E, Voce P, et al. Indoleamine 2, 3-dioxygenase 1 (IDO1) is up-regulated in thyroid carcinoma and drives the development of an immunosuppressant tumor microenvironment [J]. *J Clin Endocrinol Metab*, 2014, **99**(5): E832-840.
- [13] Spranger S, Spaapen RM, Zha Y, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8⁺ T cells [J]. *Sci Transl Med*, 2013, **5**(200): 200ra116.
- [14] Dey S, Sutanto-Ward E, Kopp KL, et al. Peptide vaccination directed against IDO1-expressing immune cells elicits CD8⁺ and CD4⁺ T-cell-mediated antitumor immunity and enhanced anti-PD1 responses [J]. *J Immunother Cancer*, 2020, **8**(2): e000605.
- [15] Lou Q, Liu R, Yang X, et al. miR-448 targets IDO1 and regulates CD8⁺ T cell response in human colon cancer [J]. *J Immunother Cancer*, 2019, **7**(1): 210.
- [16] Wei C, Mei J, Tang L, et al. 1-Methyl-tryptophan attenuates regulatory T cells differentiation due to the inhibition of estrogen-IDO1-MRC2 axis in endometriosis [J]. *Cell Death Dis*, 2016, **7**(12): e2489.
- [17] Chon SY, Hassanain HH, Gupta SL. Cooperative role of interferon regulatory factor 1 and p91 (STAT1) response elements in interferon-gamma-inducible expression of human indoleamine 2, 3-dioxygenase gene [J]. *J Biol Chem*, 1996, **271**(29): 17247-17252.
- [18] Li F, Zhang R, Li S, et al. IDO1: An important immunotherapy target in cancer treatment [J]. *Int Immunopharmacol*, 2017, **47**: 70-77.
- [19] Xiang Z, Zhou Z, Song S, et al. Dexamethasone suppresses immune evasion by inducing GR/STAT3 mediated downregulation of PD-L1 and IDO1 pathways [J]. *Oncogene*, 2021, **40**(31): 5002-5012.
- [20] Hennequart M, Pilotte L, Cane S, et al. Constitutive IDO1 expression in human tumors is driven by cyclooxygenase-2 and mediates intrinsic immune resistance [J]. *Cancer Immunol Res*, 2017, **5**(8): 695-709.
- [21] Muller AJ, DuHadaway JB, Donover PS, et al. Inhibition of indoleamine 2, 3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy [J]. *Nat Med*, 2005, **11**(3): 312-319.
- [22] Vacchelli E, Aranda F, Eggermont A, et al. Trial watch: IDO inhibitors in cancer therapy [J]. *Oncoimmunology*, 2014, **3**(10): e957994.
- [23] Bahraoui E, Serrero M, Planes R. HIV-1 Tat-TLR4/MD2 interaction drives the expression of IDO-1 in monocytes derived dendritic cells through NF-kappaB dependent pathway [J]. *Sci Rep*, 2020, **10**(1): 8177.
- [24] Li H, Deng CQ, Chen BY, et al. Total saponins of *Panax notoginseng* modulate the expression of caspases and attenuate apoptosis in rats following focal cerebral ischemia-reperfusion [J]. *J Ethnopharmacol*, 2009, **121**(3): 412-418.
- [25] Wen XD, Wang CZ, Yu C, et al. *Panax notoginseng* attenuates experimental colitis in the azoxymethane/dextran sulfate sodium mouse model [J]. *Phytother Res*, 2014, **28**(6): 892-898.
- [26] Zhang J, Ding L, Wang B, et al. Notoginsenoside R1 attenuates experimental inflammatory bowel disease via pregnane X receptor activation [J]. *J Pharmacol Exp Ther*, 2015, **352**(2): 315-324.
- [27] Zhou Z, Wang J, Song Y, et al. *Panax notoginseng* saponins attenuate cardiomyocyte apoptosis through mitochondrial pathway in natural aging rats [J]. *Phytother Res*, 2018, **32**(2): 243-250.
- [28] Hu S, Liu T, Wu Y, et al. *Panax notoginseng* saponins suppress lipopolysaccharide-induced barrier disruption and monocyte adhesion on bEnd. 3 cells via the opposite modulation of Nrf2 antioxidant and NF-kappaB inflammatory pathways [J]. *Phytother Res*, 2019, **33**(12): 3163-3176.
- [29] Lu QG, Zeng L, Li XH, et al. Protective effects of *Panax notoginseng* saponin on dextran sulfate sodium-induced colitis in rats through phosphoinositide-3-kinase protein kinase B signaling pathway inhibition [J]. *World J Gastroenterol*, 2020, **26**(11): 1156-1171.
- [30] Yuan CS, Xie JT, Zhang B, et al. Chemopreventive effects of *Panax notoginseng* and its major constituents on SW480 human colorectal cancer cells [J]. *Int J Oncol*, 2007, **31**: 1149-1156.
- [31] Lee CY, Hsieh SL, Hsieh S, et al. Inhibition of human colorectal cancer metastasis by notoginsenoside R1, an important compound from *Panax notoginseng* [J]. *Oncol Rep*, 2017, **37**(1): 399-407.
- [32] Xu C, Liu T, Liu H et al. *Panax notoginseng* saponins radiosensitize colorectal cancer cells by regulating the SNHG6/miR-137 axis [J]. *RSC Advances*, 2019, **9**(66): 38558-38567.
- [33] Wang CZ, Luo X, Zhang B, et al. Notoginseng enhances anticancer effect of 5-fluorouracil on human colorectal cancer cells [J]. *Cancer Chemother Pharmacol*, 2007, **60**(1): 69-79.
- [34] Kleiman NI, Friedman DL, Sabato GD. Preparation of single-cell suspensions from lymphoid organs [J]. *Methods Enzymol*, 1984, **108**: 43-49.
- [35] Thaker AI, Shaker A, Rao MS, et al. Modeling colitis-associated cancer with azoxymethane (AOM) and dextran sulfate sodium (DSS) [J]. *J Vis Exp*, 2012(67): 4100.
- [36] Wei TT, Lin YT, Tseng RY, et al. Prevention of colitis and colitis-associated colorectal cancer by a novel polypharmacological histone deacetylase inhibitor [J]. *Clin Cancer Res*, 2016, **22**(16): 4158-4169.
- [37] Maloy KJ, Salaun L, Cahill R, et al. CD4⁺ CD25⁺ T_R cells suppress innate immune pathology through cytokine-dependent mechanisms [J]. *J Exp Med*, 2003, **197**(1): 111-119.

- [38] Oh SA, Li MO. TGF-beta: guardian of T cell function [J]. *J Immunol*, 2013, **191**(8): 3973-3979.
- [39] Jiang GM, He YW, Fang R, *et al.* Sodium butyrate down-regulation of indoleamine 2,3-dioxygenase at the transcriptional and post-transcriptional levels [J]. *Int J Biochem Cell Biol*, 2010, **42**(11): 1840-1846.
- [40] Huang KC, Chiang SF, Chen WT, *et al.* Decitabine augments chemotherapy-induced PD-L1 upregulation for PD-L1 blockade in colorectal cancer [J]. *Cancers (Basel)*, 2020, **12**(2): 462.
- [41] Geng F, Wang Z, Yin H, *et al.* Molecular targeted drugs and treatment of colorectal cancer: recent progress and future perspectives [J]. *Cancer Biother Radiopharm*, 2017, **32**(5): 149-160.
- [42] Kishore C, Bhadra P. Current advancements and future perspectives of immunotherapy in colorectal cancer research [J]. *Eur J Pharmacol*, 2021, **893**: 173819.
- [43] Wang S, Wang X, Zhang Y, *et al.* Efficacy of Chinese herbal injections combined with fluoropyrimidine and oxaliplatin-based chemotherapy for advanced colorectal cancer: a protocol for systematic review and meta-analysis of randomized controlled trials [J]. *Medicine (Baltimore)*, 2020, **99**(52): e23550.
- [44] Ooki A, Shinozaki E, Yamaguchi K. Immunotherapy in colorectal cancer: current and future strategies [J]. *J Anus Rectum Colon*, 2021, **5**(1): 11-24.
- [45] Iwasaki T, Kohashi K, Toda Y, *et al.* Association of PD-L1 and IDO1 expression with JAK-STAT pathway activation in soft-tissue leiomyosarcoma [J]. *J Cancer Res Clin Oncol*, 2021, **147**(5): 1451-1463.
- [46] Prendergast GC, Malachowski WP, DuHadaway JB, *et al.* Discovery of IDO1 inhibitors: from bench to bedside [J]. *Cancer Res*, 2017, **77**(24): 6795-6811.
- [47] Yeung AW, Terentis AC, King NJ, *et al.* Role of indoleamine 2, 3-dioxygenase in health and disease [J]. *Clin Sci (Lond)*, 2015, **129**(7): 601-672.
- [48] Minhas PS, Liu L, Moon PK, *et al.* Macrophage de novo NAD⁺ synthesis specifies immune function in aging and inflammation [J]. *Nat Immunol*, 2019, **20**(1): 50-63.
- [49] Huynh DTN, Baek N, Sim S, *et al.* Minor ginsenoside Rg2 and Rh1 attenuates LPS-induced acute liver and kidney damages via downregulating activation of TLR4-STAT1 and inflammatory cytokine production in macrophages [J]. *Int J Mol Sci*, 2020, **21**(18): 6656.
- [50] Meissl K, Macho-Maschler S, Muller M, *et al.* The good and the bad faces of STAT1 in solid tumours [J]. *Cytokine*, 2017, **89**: 12-20.
- [51] Pflügler S, Svinka J, Scharf I, *et al.* IDO1(+) Paneth cells promote immune escape of colorectal cancer [J]. *Commun Biol*, 2020, **3**(1): 252.
- [52] Yuasa K, Hijikata T. Distal regulatory element of the STAT1 gene potentially mediates positive feedback control of STAT1 expression [J]. *Genes Cells*, 2016, **21**(1): 25-40.

Cite this article as: LI Xue-Ming, YUAN Ding-Yi, LIU Ya-Hui, ZHU Lei, QIN Hong-Kun, YANG Yu-Bing, LI Yan, YAN Fang, WANG Ya-Jing. *Panax notoginseng* saponins prevent colitis-associated colorectal cancer via inhibition IDO1 mediated immune regulation [J]. *Chin J Nat Med*, 2022, **20**(4): 258-269.