

# TNF- $\alpha$ -mediated epithelial-to-mesenchymal transition regulates expression of immune checkpoint molecules in hepatocellular carcinoma

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**Abstract.** Hepatocellular carcinoma (HCC) is the fastest growing cause of cancer-related deaths globally. Epithelial-to-mesenchymal transition (EMT) is a cellular process that confers HCC tumor cells with the ability to evade the immune system. Immune escape in most tumors, including HCC, is controlled by immune checkpoint molecules. The aim of the present study was to investigate the association between EMT and immune checkpoint in HCC, and identify novel therapeutic targets for HCC. An *in vitro* model of reversible EMT was utilized based on cytokine tumor necrosis factor (TNF)- $\alpha$  treatment of HCC cell lines Hep3B and PLC/PRF/5. Hep3B and PLC/PRF/5 cells were treated with TNF- $\alpha$ , and the EMT status and the expression of immune checkpoint molecules was assessed by reverse transcription-quantitative PCR, western blotting and immunofluorescence. To confirm an association between EMT and immune modulators, cells were exposed to culture medium with TNF- $\alpha$  for 3 days to induce EMT, following which a reversal assay was performed. The expression of immune modulators and mesenchymal-to-epithelial transition (MET) status was investigated upon reversal of EMT. Furthermore, SurvExpress, a web-based platform was utilized to analyze survival and recurrence in a dataset of patients with HCC. TNF- $\alpha$  treatment for 3 days induced EMT in Hep3B and PLC/PRF/5 cells, as demonstrated by the downregulation of epithelial markers along with upregulation in mesenchymal markers. An EMT reversal assay was able to induce MET by increasing epithelial markers and decreasing mesenchymal markers. TNF- $\alpha$ -induced EMT led to the upregulation of immune modulators, including programmed death receptor

ligand (PD-L)1, PD-L2, CD73 and B7-H3. In contrast, reversal of EMT suppressed the expression of PD-L1, PD-L2, CD73 and B7-H3. In addition, high expression of TNF- $\alpha$  and PD-L1 in 422 patients with HCC was associated with poor overall survival. The coordinate expression of TNF- $\alpha$  with PD-L2 in this patient cohort was associated with increased HCC recurrence. In conclusion, the present study demonstrated a close association between immune modulator expression and EMT induction/reversal driven by TNF- $\alpha$ .

## Introduction

Hepatocellular carcinoma (HCC) is a primary liver malignancy accounting for 80-90% of all liver cancer (1). According to the latest global cancer data, the incidence of HCC has rapidly risen with ~782,000 deaths in 2018 (2). The majority of HCC is resistant to treatment as it is often diagnosed at advanced stages of disease, so the overall median survival is <1 year (3). To the best of the authors' knowledge, sorafenib and levatinib are the only first-line systemic therapeutic agents available for the treatment of advanced HCC. Several second-line drugs, including regorafenib, ramucirumab and cabozantinib, have been approved for patients with HCC who have previously been treated with sorafenib, but the overall survival (OS) rate remains poor (4,5).

Immune checkpoint blockade therapies have emerged as potential treatments in cancer immunotherapy (6). The cytotoxic T-cell mediated immune response is accompanied by co-stimulatory signals and co-inhibitory signals or immune checkpoints (7,8). The immune checkpoint molecules are often overexpressed or activated in the tumor microenvironment, thus resulting in immune evasion by tumor cells (7-9). Immune checkpoint inhibitors (ICIs) that block programmed cell death protein-1 (PD-1) or its ligand, programmed death receptor ligand (PD-L)1, have shown therapeutic potential in several cancer types, including HCC (10,11). Anti-PD-1 ICIs, nivolumab and pembrolizumab, have been approved by The Food and Drug Administration for the treatment of HCC in patients where treatment using sorafenib has failed (12). There are a number of ongoing clinical trials on ICIs targeting PD-1 (nivolumab and pembrolizumab) and PD-L1 (atezolizumab) in HCC (7,13). Due to the success of anti-PD-1/PD-L1

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immunotherapy clinical studies, understanding the underlying mechanisms regulating PD-L1 expression has attracted increasing attention. Previous research by the authors and other researchers demonstrated an increased expression of other immune checkpoint molecules in HCC (8,14). However, the mechanisms regulating the expression of these and other immune checkpoint molecules in HCC remains unclear (8).

A key process in HCC progression is epithelial-to-mesenchymal transition (EMT). EMT is a complex molecular and cellular process that allows epithelial cells to gain mesenchymal features, including migration, invasiveness and increased resistance to immune evasion (15,16). Mesenchymal-to-epithelial transition (MET) is the reverse process of EMT that offers phenotypic plasticity for conversion of mesenchymal cells to epithelial derivatives (15). EMT status is closely associated with cancer metastasis, stemness, immune escape and drug resistance in HCC (17,18). It was previously identified that EMT is involved in the regulation of PD-L1 in several cancer types, including breast cancer (19,20), lung cancer (17,21), pancreatic cancer (22), esophageal cancer (23) and salivary adenoid cystic carcinoma (24). The authors previously demonstrated that EMT is associated with PD-L1 expression in patients with HCC (8).

In the present study, it was hypothesized that EMT is associated with the ability of HCC tumor cells to escape detection and destruction by the immune response, by regulating the expression of immune checkpoint molecules, such as PD-L1. In the present study, the changes in immune checkpoint expression during the EMT process were examined by incorporating a reversible model of EMT based on tumor necrosis factor (TNF)- $\alpha$  treatment of the HCC cell lines Hep3B and PLC/PRF/5. TNF- $\alpha$  was selected as an inducer of EMT, as it has been previously demonstrated to play an important role in promoting EMT in HCC (25). This *in vitro* system may provide an improved understanding of the modulation of immune checkpoints during both EMT and MET.

## Materials and methods

**Cell culture and reagents.** The human HCC cell line Hep3B was provided by Professor V. Nathan Subramaniam, The Queensland University of Technology. The human HCC cell line PLC/PRF/5 was purchased from CellBank Australia (cat. no. 85061113). Both cell lines were mycoplasma-tested using the MycoAlert™ mycoplasma detection kit (Lonza Group, Ltd.) and cultured in DMEM (Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.), and incubated at 37°C under a humidified atmosphere with 5% CO<sub>2</sub> in air (26). The cytokine TNF- $\alpha$  was purchased from PeproTech, Inc.

**EMT reversal assay.** An EMT reversal assay was performed to determine the association between EMT and immune checkpoint expression. Firstly, EMT was induced by using culture medium with 20 ng/ml TNF- $\alpha$  for 3 days at 37°C and then reversal of EMT was induced by changing the culture medium without TNF- $\alpha$  for the next 3 days.

**RNA extraction and cDNA synthesis.** RNA was isolated from Hep3B and PLC/PRF/5 cells, as previously described, using

Table I. List of primers for reverse transcription-quantitative PCR.

Gene	Primer sequence, 5'-3'
<i><math>\beta</math>-actin</i>	F: CCAACCGCGAGAAGATGA R: CCAGAGGCGTACAGGGATAG
<i>E-cadherin</i>	F: AGGCCAAGCAGCAGTACATT R: ATTCACATCCAGCACATCCA
<i>N-cadherin</i>	F: TCCTTGCTTCTGACAATGGA R: TTCGCAAGTCTCTGCCTCTT
<i>Occludin</i>	F: TAGTCAGATGGGGGTGAAGG R: CATTATGATGAGCAGCCCC
<i>Vimentin</i>	F: CTTCAGAGAGAGGAAGCCGA R: ATTCCACTTTGCGTTCAAGG
<i>Snai2</i>	F: TGGTTGCTTCAAGGACACAT R: GTTGCAAGTGAAGGCAAGAA
<i>Fibronectin</i>	F: CAGTGGGAGACCTCGAGAAG R: TCCCTCGGAACATCAGAAAC
<i>PD-L1</i>	F: TGCCGACTACAAGCGAATTACTG R: CTGCTTGTCCAGATGACTTCGG
<i>PD-L2</i>	F: ACCGTGAAAGAGCCACTTTG R: GCGACCCCATAGATGATTATGC
<i>CD73</i>	F: TTGGAAATTTGGCCTCTTTG R: ACTTCATGAACGCCCTGC
<i>B7-H3</i>	F: CTGGCTTTCGTGTGCTGGAGAA R: GCTGTCAGAGTGTTTCAGAGGC
<i>VISTA</i>	F: AGATGCACCATCCAAGTGTGTGG R: AGGCAGAGGATTCTACGATGC
<i>VTCN1</i>	F: CTCACAGATGCTGGCACCTACA R: GCAAGGTCTCTGAGCTGGCATT

*PD-L*, programmed death receptor ligand; *Snai2*, snail family transcriptional repressor 2; F, forward; R, reverse; *VTCN1*, V-set domain-containing T-cell activation inhibitor 1; *VISTA*, V-domain immunoglobulin suppressor of T-cell activation.

Isolate II Bioline RNA synthesis kit (Bioline), according to the manufacturer's protocol (26). The quantity and purity of RNA was confirmed using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized by reverse transcribing 1  $\mu$ g RNA into cDNA using the Bioline SensiFAST cDNA synthesis kit (Bioline) with the following thermocycling conditions: Primer annealing at 25°C for 10 min, reverse transcription at 42°C for 15 min, inactivation at 85°C for 5 min and a final extension 4°C for 30 min.

**Reverse transcription-quantitative (RT-q)PCR.** RT-qPCR was performed using SensiFast™ SYBR® Lo-ROX kit (Bioline) on a ViiA7 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), as previously described (26). A three-step cycle procedure was applied with 40 cycles of the following conditions: 95°C for 5 sec, 63°C for 20 sec and 75°C for 20 sec.  $\beta$ -actin was used as an internal control. The primer sequences used are listed in Table I. Data were analyzed using

Table II. List of antibodies.

A, Primary antibodies

Name	Cat. no.	Manufacturer	Dilution	
			Western blotting	Immuno-fluorescence
E-cadherin	ab76055	Abcam	1:1,000	1:100
N-cadherin	SC-53488	Santa Cruz Biotechnology, Inc.	1:200	-
Occludin	SC-133255	Santa Cruz Biotechnology, Inc.	1:200	1:50
Vimentin	ab92547	Abcam	1:1,000	1:600
CD73	ab133582	Abcam	1:250	-
CD73	ab175396	Abcam	-	1:100
PD-L1	ab238697	Abcam	2 $\mu$ g/ml	2 $\mu$ g/ml
B7-H3	14058S	Cell Signaling Technology, Inc.	1:1,000	-
PD-L2	82723S	Cell Signaling Technology, Inc.	1:1,000	-
GAPDH	MAB376	EMD Millipore	1:100,000	-

B, Secondary antibody

Name	Cat. no.	Manufacturer	Dilution	
			Western blotting	Immuno-fluorescence
Goat anti-mouse HRP	6421040	Invitrogen; Thermo Fisher Scientific, Inc.	1:50,000	-
Goat anti-rabbit HRP	656120	Invitrogen; Thermo Fisher Scientific, Inc.	1:50,000	-
Alexafluor 488 anti-mouse	A21202	Thermo Fisher Scientific, Inc.	-	1:200
Alexafluor 594 anti-rabbit	A21207	Thermo Fisher Scientific, Inc.	-	1:200

PD-L, programmed death receptor ligand; HRP, horseradish peroxidase; -, not applicable.

the  $2^{-\Delta\Delta C_q}$  method where  $\beta$ -actin was assigned as the house-keeping gene. The results are expressed as relative mRNA expression to the control (27).

**Western blot analysis.** Cells were seeded ( $2 \times 10^5$  cells/well) in 6-well plates and subsequently lysed using RIPA buffer (Thermo Fisher Scientific, Inc.) with complete protease inhibitors and PhosSTOP™ phosphatase inhibitors (both purchased from Roche Diagnostics) on ice to extract the total protein. The total protein was measured with a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 10  $\mu$ g protein was used for 4% SDS-PAGE, following which proteins were transferred to a PVDF membrane. After blocking with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 h, the membranes were incubated with primary antibodies at 4°C overnight. An enhanced chemiluminescence reagent, SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.), was used to detect the protein adhering to the membranes following incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. All antibodies used are listed in Table II, including the dilutions, cat. nos. and suppliers. GAPDH was selected as the housekeeping control in each group. The images were captured with ImageQuant™ LAS 500 and quantified with Image Studio™ Lite v5.2 software (LI-COR Biosciences).

**Immunofluorescence.** Cells ( $5 \times 10^3$  cells/well) were cultured in 8-well tissue culture treated chamber slides. Cells were washed with 1X PBS and then fixed with 4% paraformaldehyde (Thermo Fisher Scientific, Inc.) at room temperature for 20 min followed by treatment with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA). The cells were then blocked with 5% FBS at room temperature for 1 h, followed by overnight incubation with primary antibodies at 4°C. The cells were incubated with secondary antibodies at room temperature for 1 h, followed by 10 min incubation with DAPI (Thermo Fisher Scientific, Inc.) at room temperature. All antibodies used are listed in Table II, including the dilutions, cat. nos. and suppliers. The slides were then mounted in ProLong® Diamond (Thermo Fisher Scientific, Inc.) for observation with a Nikon C2 confocal microscope (magnification, x40), and captured and analyzed with NIS-Elements-AR software (version 5.01.00; Nikon Corporation).

**SurvExpress bioinformatics tool.** An online tool, SurvExpress, was used to generate survival analysis using datasets of patients with HCC, as previously described (8,28). A total of six publically available datasets of patients with HCC were used: Hoshida Golub Liver GSE10143 (162 patients), Tsuchiya Rusyn Liver GSE17856 (95 patients), Hoshida Golub Liver GSE10186 (118 patients), Liver Hepatocellular Carcinoma The Cancer Genome Atlas (TCGA; 12 patients), TCGA-Liver-Cancer

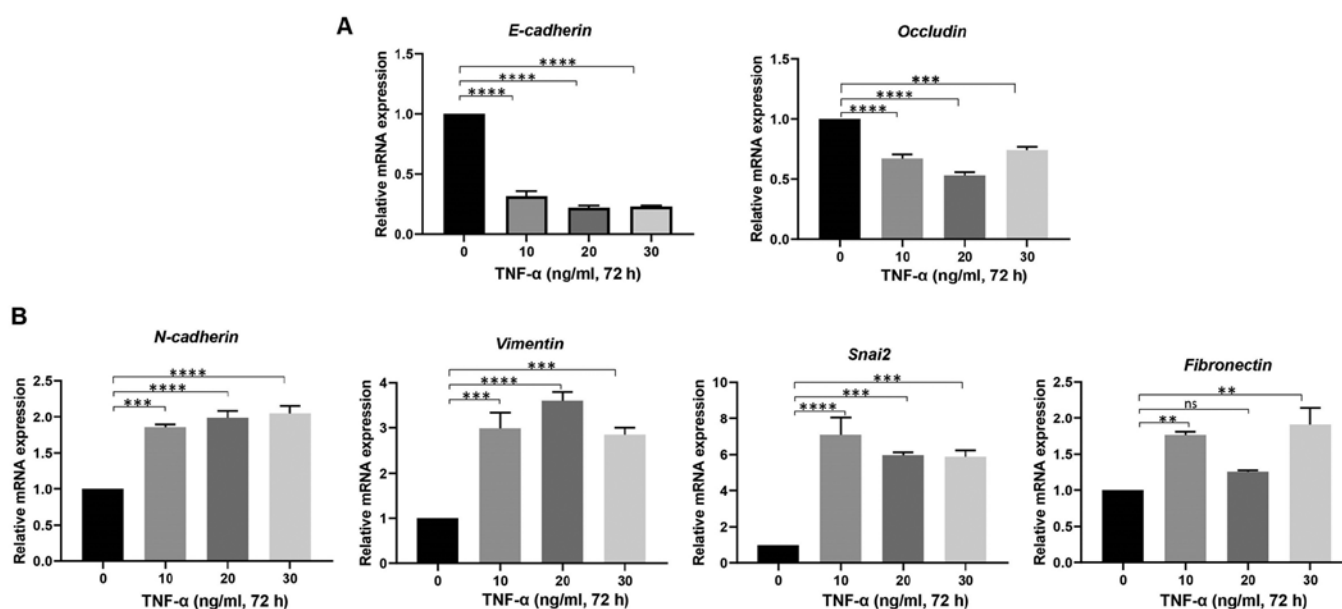


Figure 1. TNF- $\alpha$  induces epithelial-to-mesenchymal transition in Hep3B cells at various doses. Reverse transcription-quantitative PCR analysis demonstrated (A) lower expression of *E-cadherin* and *Occludin*, and (B) higher expression of *N-cadherin*, *Vimentin*, *Snai2* and *Fibronectin* upon treatment with TNF- $\alpha$  at concentrations of 0, 10, 20 and 30 ng/ml for 72 h. n=3. \*\*P<0.01, \*\*\*P<0.005, \*\*\*\*P<0.001. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; *Snai2*, snail family transcriptional repressor 2; ns, not significant.

(422 patients) and LIHC-TCGA-Liver hepatocellular carcinoma June 2016 (361 patients) (8,28-31). The gene expression of immune checkpoint molecules in combination with TNF- $\alpha$  were examined and their association with the survival of patients was analyzed (Cox regression analyses) using the patient database with patient survival information (8). The survival times were estimated using Kaplan-Meier curves (8).

**Statistical analysis.** All experiments were repeated at least three times and representative results are presented. Statistical analyses were performed using Prism software version 8.00 (GraphPad Software, Inc.). Data for dose concentration and time course experiments were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test. Comparisons of TNF- $\alpha$ -induced EMT and MET following reversal assay were performed using ANOVA followed by Sidak's multiple comparisons test. Gene expression differences between control and TNF- $\alpha$  treated cells were analyzed using Student's t-test. The results are presented as the mean  $\pm$  SEM. Error bars indicate SEM. P<0.05 was considered to indicate a statistically significant difference.

For survival analyses using SurvExpress, a log-rank test was used for testing the P-value of survival curves, and deviance residuals were applied for the correlation coefficient (8,32). Moreover, the hazard ratio (HR) between the groups were estimated using another Cox model (8).

## Results

**TNF- $\alpha$  induces EMT in human HCC cells.** Several cytokines are known to induce EMT in HCC cells, including TNF- $\alpha$ . In order to induce EMT, Hep3B and PLC/PRF/5 cells were treated with various concentrations of TNF- $\alpha$  for 72 h to assess EMT induction. EMT induced by TNF- $\alpha$  in Hep3B cells was determined by downregulation of epithelial markers

(*E-cadherin* and *Occludin*) and upregulation of mesenchymal markers [*N-cadherin*, *Vimentin*, snail family transcriptional repressor 2 (*Snai2*) and *Fibronectin*]. The optimal EMT effects of TNF- $\alpha$  were observed at 20 ng/ml (Fig. 1). Thus, the concentration of 20 ng/ml was selected for all further studies. Furthermore, Hep3B cells were treated with 20 ng/ml TNF- $\alpha$  at various time-points to observe optimal EMT induction. The qPCR results showed that TNF- $\alpha$  treatment at various time-points was able to induce EMT in Hep3B cells (Fig. 2). At 72 h, robust EMT marker changes were observed; therefore, this time-point was selected for further study. Similarly, robust EMT in PLC/PRF/5 cells treated with 20 ng/ml of TNF- $\alpha$  at 72 h was observed. EMT induction was demonstrated by the downregulation of epithelial markers (*E-cadherin* and *Occludin*) and an upregulation of mesenchymal markers (*N-cadherin* and *Vimentin*; Fig. S1). No expression of *Snai2* and *Fibronectin* was detected in this cell line. The ability of TNF- $\alpha$  to induce EMT in HCC was further validated at the protein level by western blot analysis in Hep3B (Fig. 3A) and PLC/PRF/5 (Fig. 3B) cells.

**TNF- $\alpha$ -mediated EMT upregulates immune checkpoint expression.** To examine whether TNF- $\alpha$ -mediated EMT influenced the expression of immune modulators, Hep3B and PLC/PRF/5 cells were treated with 20 ng/ml TNF- $\alpha$  for 72 h. The cells were then evaluated for expression of immune modulators *PD-L1*, *PD-L2*, *CD73*, *B7-H3*, V-set domain-containing T-cell activation inhibitor 1 (*VTCN1*) and V-domain immunoglobulin suppressor of T-cell activation (*VISTA*). These immune regulators were assessed as our group previously demonstrated that these immune checkpoint molecules are associated with a poor prognosis in patients with HCC (8). The qPCR results revealed upregulation of four immune modulators (*PD-L1*, *PD-L2*, *CD73* and *B7-H3*) and downregulation of two immune modulators (*VTCN1* and *VISTA*) upon treatment

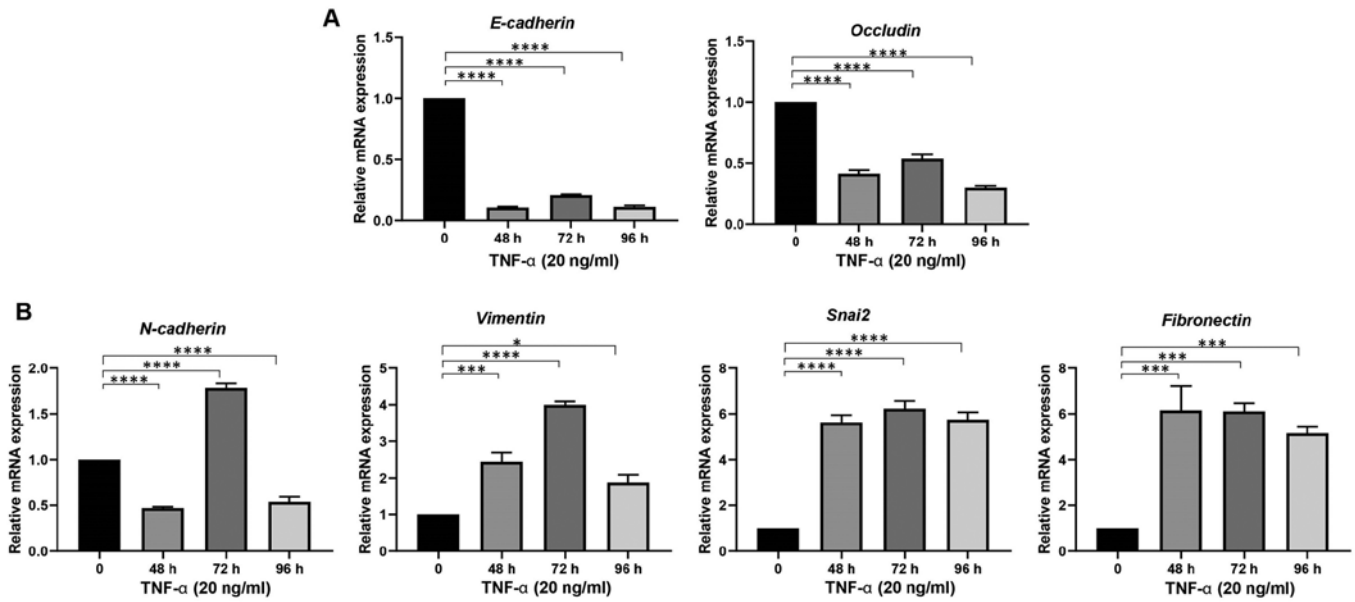


Figure 2. TNF- $\alpha$  induces epithelial-to-mesenchymal transition in Hep3B cells at various time-points. Reverse transcription-quantitative PCR analysis demonstrated (A) lower expression of *E-cadherin* and *Occludin*, and (B) higher expression of *N-cadherin*, *Vimentin*, *Snai2* and *Fibronectin* upon treatment with 20 ng/ml TNF- $\alpha$  at time-points of 48, 72 and 96 h. n=3. \*P<0.05, \*\*\*P<0.005, \*\*\*\*P<0.001. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; *Snai2*, snail family transcriptional repressor 2.

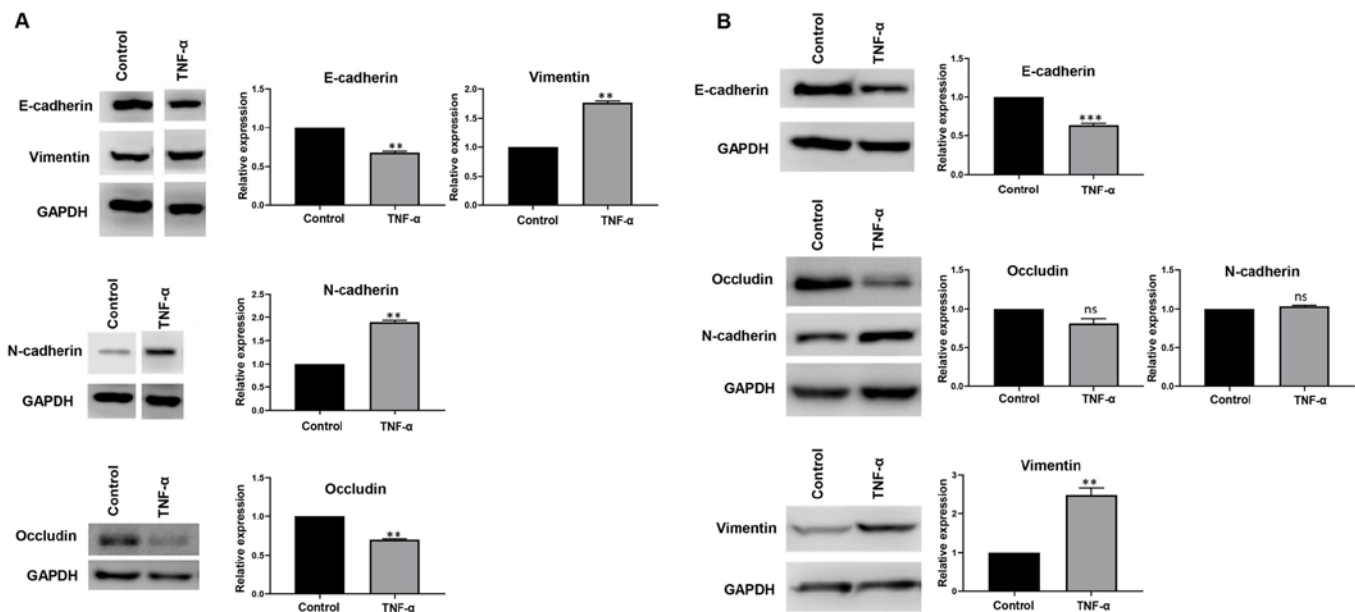


Figure 3. TNF- $\alpha$  induces epithelial-to-mesenchymal transition in hepatocellular carcinoma cells. Western blot analysis demonstrated lower expression of *E-cadherin* and *Occludin*, and higher expression of *N-cadherin* and *Vimentin* in (A) Hep3B and (B) PLC/PRF/5 cells upon treatment with 20 ng/ml TNF- $\alpha$  for 72 h. n=3. \*\*P<0.01, \*\*\*P<0.005 vs. control. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; ns, not significant.

with TNF- $\alpha$  in Hep3B cells (Fig. 4A and B). The upregulation of immune checkpoints by TNF- $\alpha$ -mediated EMT in Hep3B cells was validated at the protein level by western blot analysis (Fig. 4C).

Similarly, upregulation of immune modulators *PD-L1*, *CD73* and *B7-H3* was observed upon treatment with TNF- $\alpha$  in PLC/PRF/5 cells (Fig. 5A). *PD-L2*, *VTCN1* and *VISTA* were not detected in PLC/PRF/5 cells. This upregulation of immune modulators by TNF- $\alpha$  in PLC/PRF/5 cells was further confirmed by western blot analysis (Fig. 5B). This

demonstrated that TNF- $\alpha$  has a potentially important role in modulating immune checkpoints and EMT in HCC.

**Reversal of TNF- $\alpha$ -mediated EMT reverses immune checkpoint expression.** In order to assess the association between TNF- $\alpha$ -mediated EMT and immune modulator expression, an EMT reversal assay was performed. The induction of MET was evidenced by the increase in epithelial markers (*E-cadherin* and *Occludin*) and a decrease in mesenchymal markers (*N-cadherin*, *Vimentin*, *Snai2* and *Fibronectin*) in

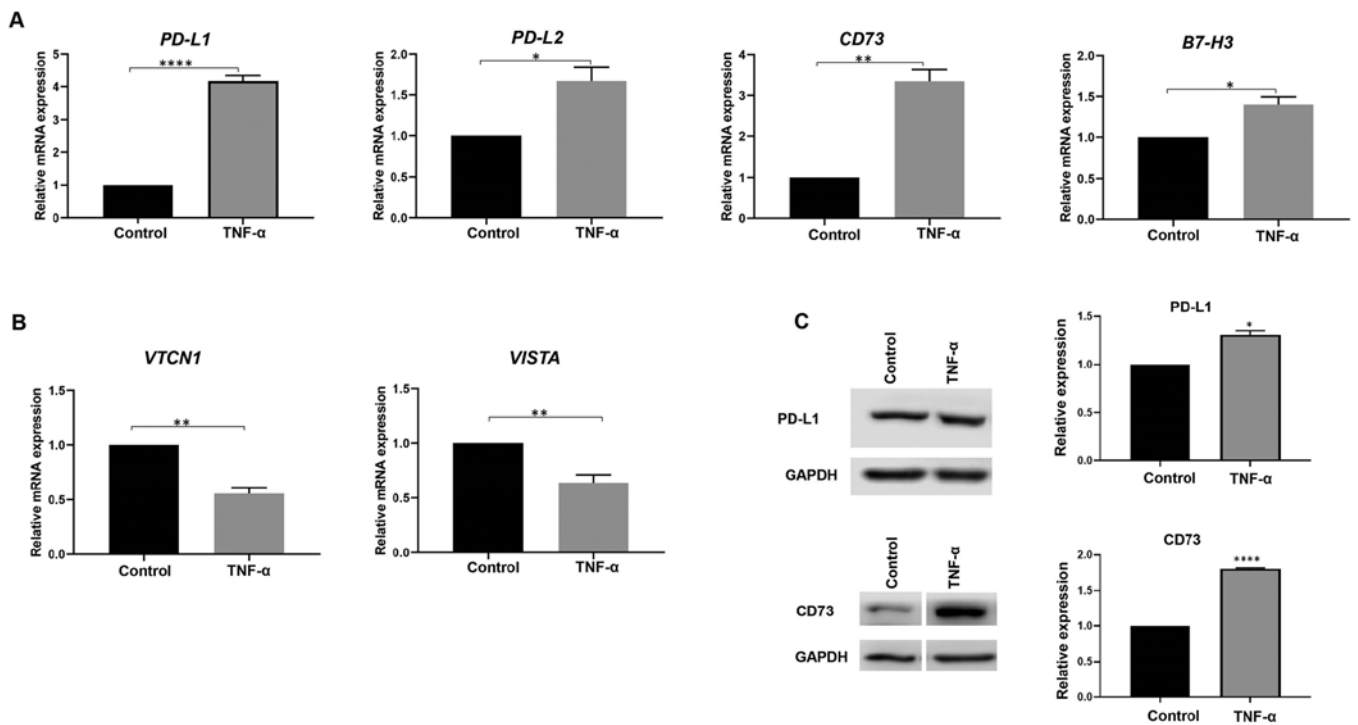


Figure 4. TNF- $\alpha$ -induced epithelial-to-mesenchymal transition regulates expression of immune checkpoint molecules in Hep3B cells. Reverse transcription-quantitative PCR analysis demonstrated (A) higher expression of *PD-L1*, *PD-L2*, *CD73* and *B7-H3*, and (B) lower expression of *VTCN1* and *VISTA* upon treatment with 20 ng/ml TNF- $\alpha$  for 72 h.  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ , \*\*\*\* $P<0.001$ . (C) Western blot analysis demonstrated upregulation of PD-L1 and CD73 in Hep3B cells upon treatment with 20 ng/ml TNF- $\alpha$  for 72 h.  $n=3$ . \* $P<0.05$ , \*\*\*\* $P<0.001$  vs. control. GAPDH was used as the loading control. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; *VTCN1*, V-set domain-containing T-cell activation inhibitor 1; *VISTA*, V-domain immunoglobulin suppressor of T-cell activation; PD-L, programmed death receptor ligand.

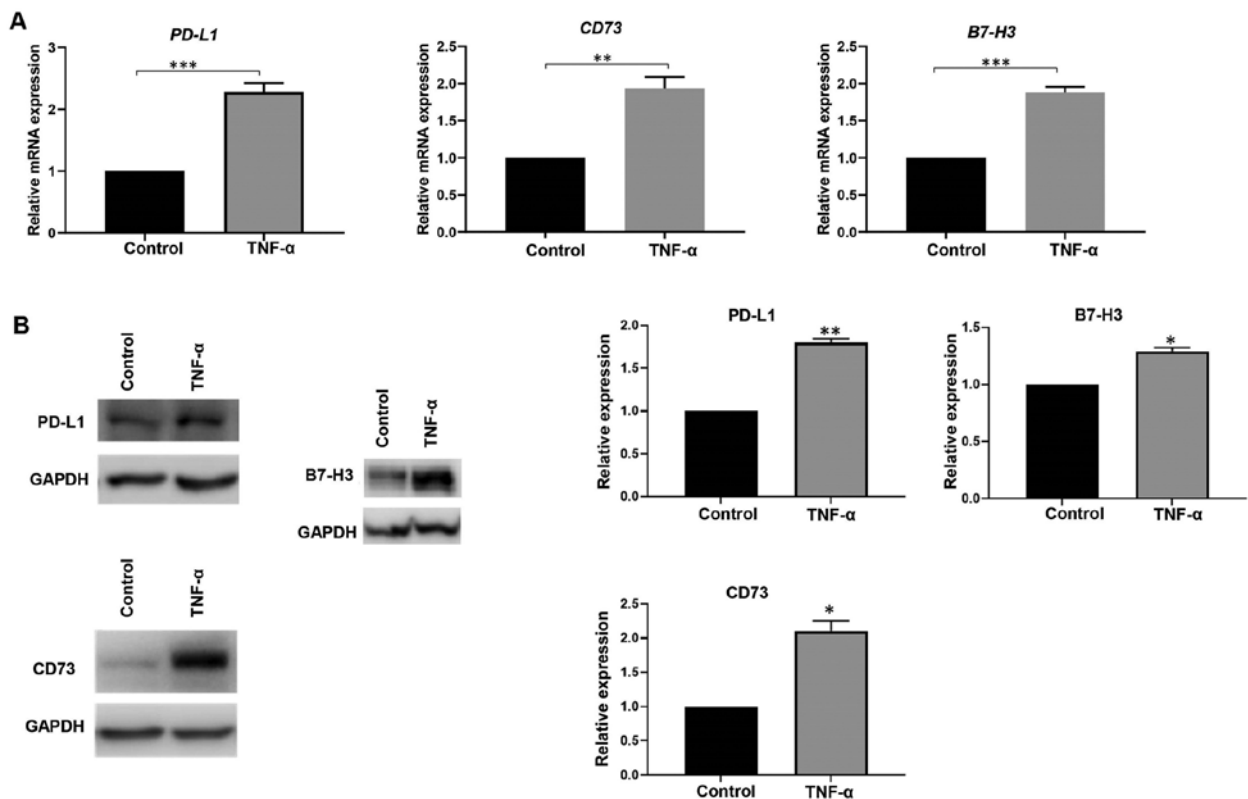


Figure 5. TNF- $\alpha$ -induced epithelial-to-mesenchymal transition regulates expression of immune checkpoint molecules in PLC/PRF/5 cells. (A) Reverse transcription-quantitative PCR analysis demonstrated higher expression of *PD-L1*, *CD73* and *B7-H3* in PLC/PRF/5 cells upon treatment with 20 ng/ml TNF- $\alpha$  for 72 h.  $n=3$ . \*\* $P<0.01$ , \*\*\*\* $P<0.005$ . (B) Western blot analysis demonstrated upregulation of PD-L1, CD73 and B7-H3 in PLC/PRF/5 cells upon treatment with 20 ng/ml TNF- $\alpha$  for 72 h.  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$  vs. control. GAPDH was used as the loading control. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; PD-L, programmed death receptor ligand.



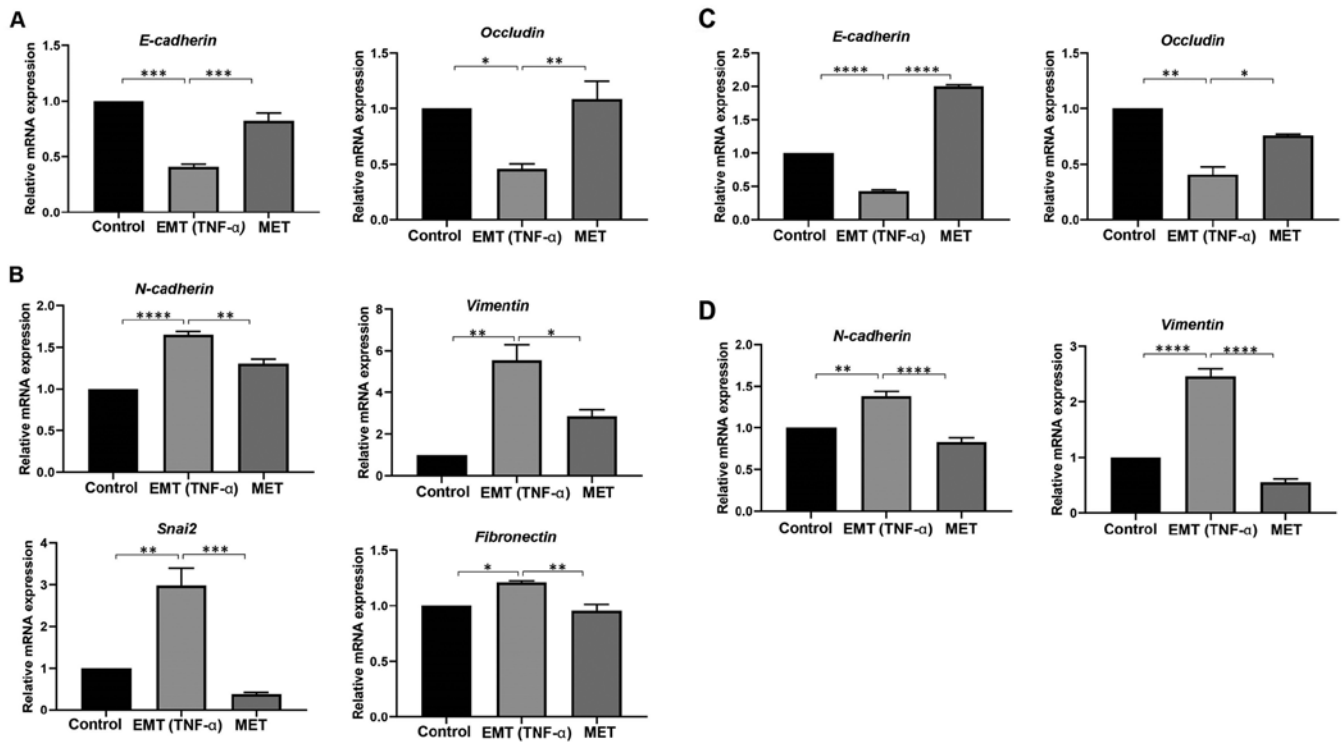


Figure 6. TNF- $\alpha$ -induced EMT in hepatocellular carcinoma cells is reversible. RT-qPCR analysis demonstrated (A) higher expression of *E-cadherin* and *Occludin*, and (B) lower expression of *N-cadherin*, *Vimentin*, *Snai2* and *Fibronectin* in TNF- $\alpha$  treated Hep3B cells following reversal assay. n=3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.005, \*\*\*\*P<0.001. RT-qPCR analysis presented (C) higher expression of *E-cadherin* and *Occludin*, and (D) lower expression of *N-cadherin* and *Vimentin* in TNF- $\alpha$  treated PLC/PRF/5 cells following reversal assay. n=3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.005, \*\*\*\*P<0.001. EMT, epithelial-to-mesenchymal transition; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; RT-qPCR, reverse transcription-quantitative PCR; *Snai2*, snail family transcriptional repressor 2; MET, mesenchymal-to-epithelial transition.

Hep3B cells (Fig. 6A and B). Similar MET was observed in PLC/PRF/5 cells, with an increase in epithelial markers (*E-cadherin* and *Occludin*) and a decrease in mesenchymal markers (*N-cadherin* and *Vimentin*; Fig. 6C and D). The changes in the EMT status observed by the reversal assay in Hep3B cells was further confirmed by immunofluorescence and western blot analysis (Fig. 7). The reversal of EMT status in the reversal assay in PLC/PRF/5 cells was also demonstrated by immunofluorescence and western blot analysis (Fig. S2).

The expression of immune checkpoints was also detected following the reversal assay. Downregulation of *PD-L1*, *PD-L2*, *CD73* and *B7-H3* was observed in Hep3B cells upon removal of TNF- $\alpha$  (Fig. 8A). Downregulation of *PD-L1*, *CD73* and *B7-H3* was additionally observed in PLC/PRF/5 cells upon removal of TNF- $\alpha$  (Fig. 8B). The reversal in immune checkpoint expression upon reversal of TNF- $\alpha$ -mediated EMT suggested that TNF- $\alpha$  is involved in regulation of immune checkpoint expression.

To further investigate the association between TNF- $\alpha$ -mediated EMT and *PD-L1* expression, immunofluorescence and western blot analysis were performed upon TNF- $\alpha$  treatment followed by the reversal assay. The immunofluorescence staining demonstrated upregulated expression of *PD-L1* and *CD73* during TNF- $\alpha$ -induced EMT and decreased expression upon reversal of EMT in Hep3B cells (Fig. 9A and B). In addition, western blot analysis showed increased expression of *PD-L1*, *CD73*, *B7-H3* and *PD-L2* during TNF- $\alpha$ -induced EMT and decreased expression upon reversal of EMT in Hep3B cells (Fig. 9C).

Furthermore, the immunofluorescence staining showed elevated expression of *PD-L1* and *CD73* during TNF- $\alpha$ -induced EMT and decreased expression upon reversal of EMT in PLC/PRF/5 cells (Fig. 10A and B). Moreover, western blot analysis showed increased expression of *PD-L1*, *CD73* and *B7-H3* during TNF- $\alpha$ -induced EMT and decreased expression upon reversal of EMT in PLC/PRF/5 cells (Fig. 10C). Overall, the present data demonstrated that TNF- $\alpha$  simultaneously induced EMT and immune checkpoint expression in HCC cells.

*Coordinate expression of TNF- $\alpha$  and immune modulators in HCC.* A total of six different HCC datasets within SurvExpress were used to analyze the recurrence free survival and OS of immune checkpoint molecules in combination with TNF- $\alpha$  in HCC patients. It was observed that coordinate expression of *PD-L1* in combination with TNF- $\alpha$  resulted in a significantly worse OS in 422 patients [HR: 1.45; confidence interval (CI): 1.03-2.02; Log-Rank Equal Curves P=0.02948; Fig. 11A]. Similarly, *PD-L2* when combined with TNF- $\alpha$  showed a significantly worse recurrence free survival (HR: 1.44; CI: 1.00-2.06; Log-Rank Equal Curves P=0.04606; Fig. 11B). There was no significant difference in recurrence free survival and OS in patients with HCC who showed coordinate expression of TNF- $\alpha$  with *CD73* or *B7-H3* (data not shown). The present data suggested a significant association between TNF- $\alpha$  and immune checkpoint expression in patients with HCC, as they showed an adverse prognosis with this profile.

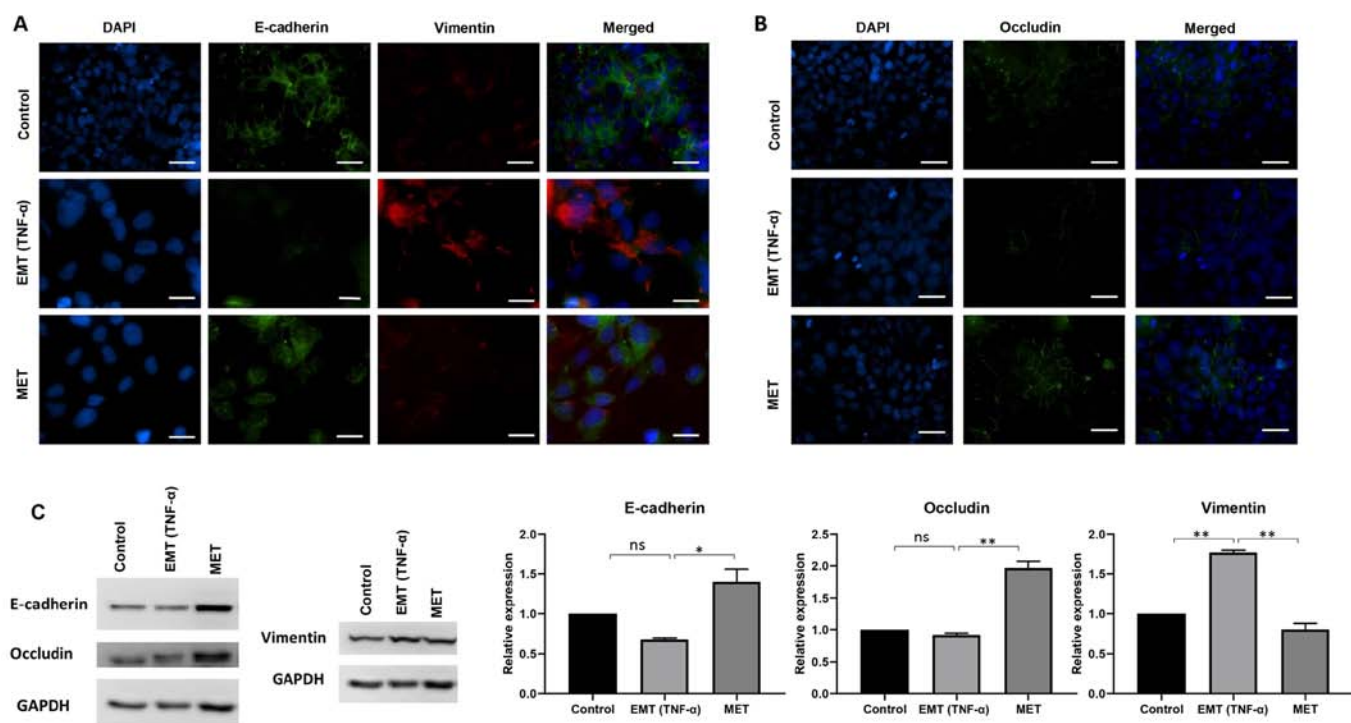


Figure 7. TNF- $\alpha$ -induced EMT in Hep3B cells is reversible. Fluorescence microscopy identified (A) suppression of E-cadherin and upregulation of Vimentin during EMT, and upregulation of E-cadherin and decreased expression of Vimentin during MET. (B) During EMT, repression of Occludin was observed and during MET, higher expression of Occludin was observed. Scale bar, 100  $\mu$ m. Magnification, x40. (C) Western blot analysis demonstrated upregulation of E-cadherin and Occludin, and downregulation of Vimentin during MET.  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ . GAPDH was used as loading control. EMT, epithelial-to-mesenchymal transition; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MET, mesenchymal-to-epithelial transition; ns, not significant.

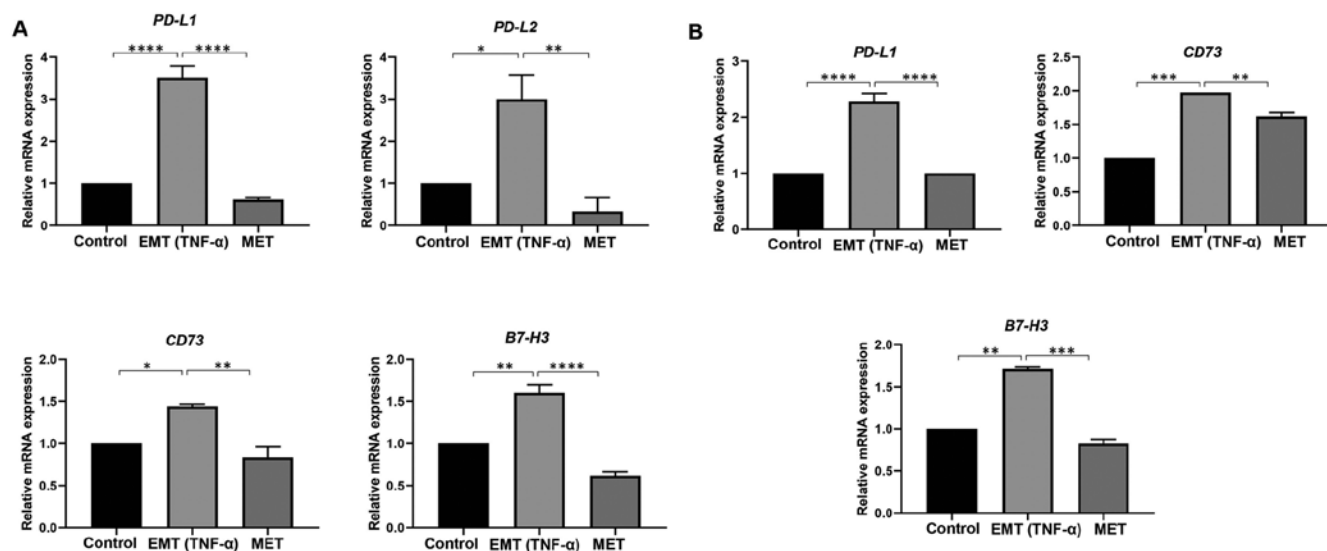


Figure 8. Reversal of EMT returns immune checkpoint expression to control levels in hepatocellular carcinoma cells. Reverse transcription-quantitative PCR analysis demonstrated (A) lower expression of immune checkpoint molecules *PD-L1*, *PD-L2*, *CD73* and *B7-H3* in EMT induced Hep3B cells and (B) lower expression of immune checkpoint molecules *PD-L1*, *CD73* and *B7-H3* in EMT induced PLC/PRF/5 cells following reversal assay.  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.005$ , \*\*\*\* $P<0.001$ . EMT, epithelial-to-mesenchymal transition; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MET, mesenchymal-to-epithelial transition; *PD-L*, programmed death receptor ligand.

## Discussion

In the present study, an association between EMT and immune checkpoint molecules in HCC was identified. It was demonstrated that TNF- $\alpha$  simultaneously induced EMT and the expression of immune checkpoint molecules PD-L1, PD-L2,

CD73 and B7-H3 in Hep3B cells, along with increased expression of PD-L1, CD73 and B7-H3 in PLC/PRF/5 cells. Moreover, it was demonstrated that EMT status is closely associated with immune checkpoint upregulation as MET induced by the reversal of TNF- $\alpha$ -mediated EMT attenuated the expression of immune checkpoints. Notably, a significant association between



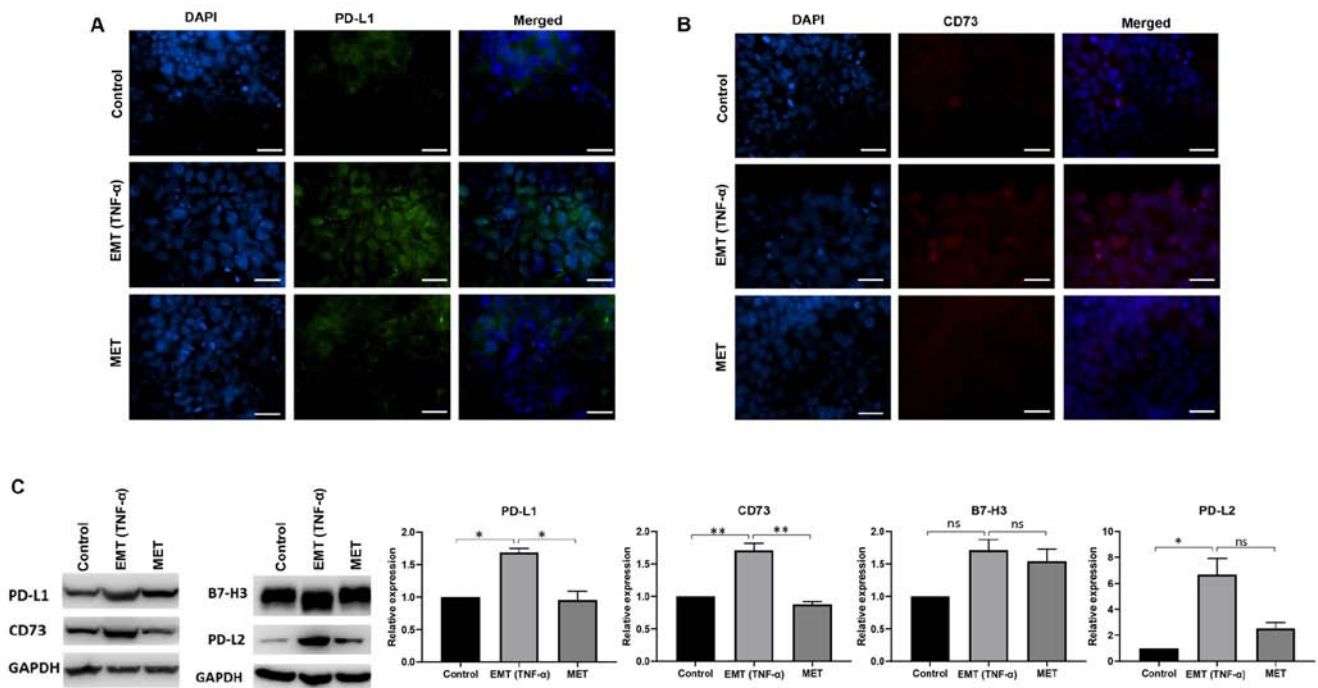


Figure 9. Reversal of EMT returns immune checkpoint expression to control levels in Hep3B cells. Fluorescence microscopy showed (A) higher expression of PD-L1 upon EMT induction and lower expression of PD-L1 following reversal assay and (B) higher expression of CD73 upon EMT induction and lower expression of CD73 following reversal assay in Hep3B cells. Scale bar, 100  $\mu$ m. Magnification, x40. (C) Western blot analysis demonstrated higher expression of PD-L1, CD73, B7-H3 and PD-L2 upon EMT induction and lower expression following reversal assay in Hep3B cells.  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ . GAPDH was used as the loading control. EMT, epithelial-to-mesenchymal transition; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MET, mesenchymal-to-epithelial transition; PD-L, programmed death receptor ligand.

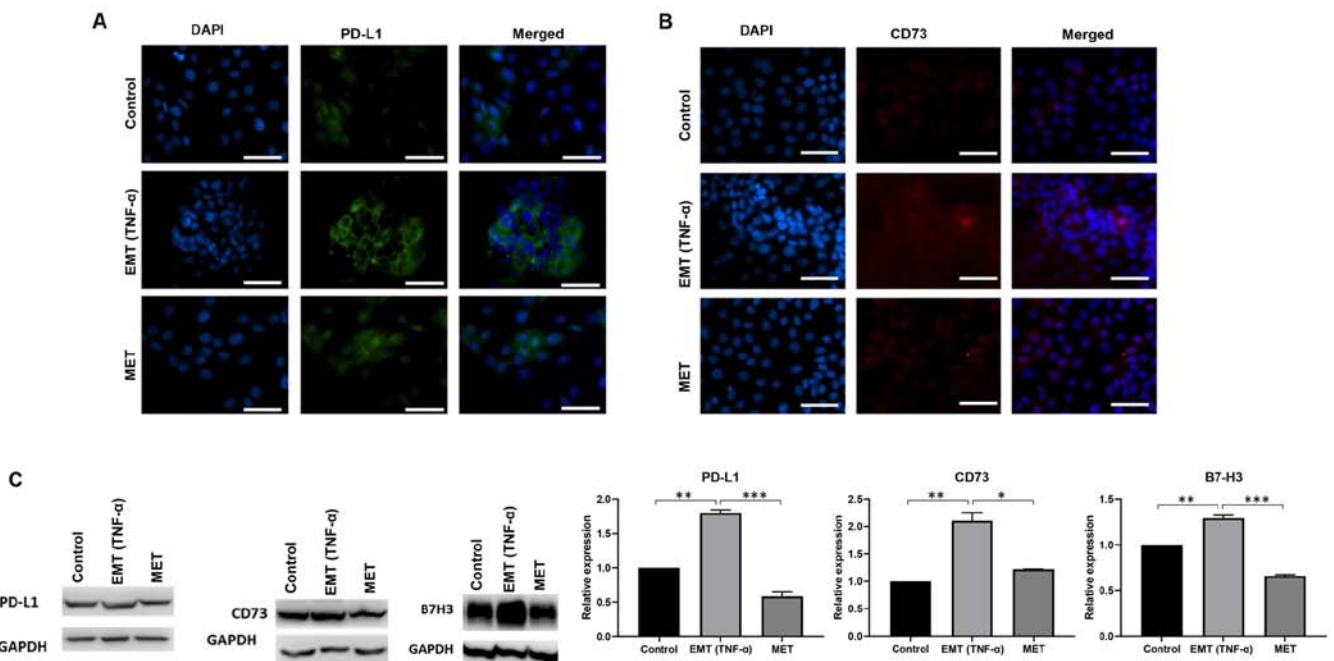


Figure 10. Reversal of EMT reverses immune checkpoint expression in PLC/PRF/5 cells. Fluorescence microscopy revealed (A) higher expression of PD-L1 upon EMT induction and lower expression of PD-L1 following reversal assay, and (B) higher expression of CD73 upon EMT induction and lower expression of CD73 following reversal assay in PLC/PRF/5 cells. Scale bar, 100  $\mu$ m. Magnification, x40. (C) Western blot analysis demonstrated higher expression of PD-L1, CD73 and B7-H3 upon EMT induction and lower expression following reversal assay in PLC/PRF/5 cells.  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.005$ . GAPDH was used as the loading control. EMT, epithelial-to-mesenchymal transition; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MET, mesenchymal-to-epithelial transition; PD-L, programmed death receptor ligand.

TNF- $\alpha$  and immune checkpoint levels was identified in patients with HCC. Patients with increased expression of both TNF- $\alpha$

and PD-L1 showed poor OS, whereas patients with high expression of TNF- $\alpha$  and PD-L2 showed high rates of recurrence.

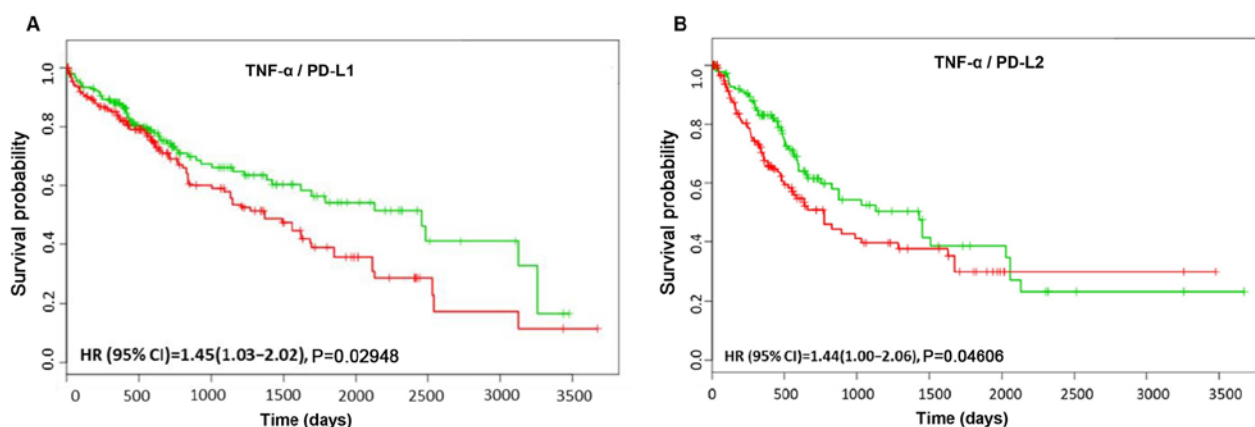


Figure 11. Association of TNF- $\alpha$  in combination with immune checkpoints and survival in patients with HCC. Kaplan-Meier survival curves for the analysis of (A) gene expression of PD-L1/TNF- $\alpha$  and overall survival, and (B) gene expression of PD-L2/TNF- $\alpha$  and recurrence free survival in samples from patients with HCC. Red curve represents the high-risk group, while green curve represents the low-risk group (8). Markers (+) represent censoring samples. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; PD-L, programmed death receptor ligand; HR, hazard ratio; HCC, hepatocellular carcinoma.

Various systems have been utilized to induce EMT in human HCC cell lines (33,34). Stimulation of HCC cells with TNF- $\alpha$  leads to the induction of EMT (35). TNF- $\alpha$  is known to induce EMT alone or in combination with other cytokines such as transforming growth factor (TGF)- $\beta$  in several cancer types, such as breast cancer, lung cancer and HCC (19,21,25). In the present study, a TNF- $\alpha$  based *in vitro* model was used to induce EMT in a reversible manner. To the best of the authors' knowledge, the present study is the first study to evaluate the role of EMT in the regulation of immune checkpoint expression in HCC.

Despite the promising results of ICIs from clinical trials, these therapies have failed in several instances due to mutations that alter immunogenicity, expression of alternative immune checkpoint molecules and dysregulated T-cell infiltration (36). Understanding the underlying molecular biological mechanisms regulating immune checkpoints may result in developing new and effective treatment strategies. In the present study it was noted that the expression of immune checkpoint molecules PD-L1, PD-L2, CD73 and B7-H3 was upregulated following TNF- $\alpha$ -induced EMT. PD-L1 is an essential and widely studied immunomodulatory ligand that is aberrantly upregulated in several cancer types, has roles in promoting tumor escape and is associated with poor prognosis (37-41). Our group and other researchers have reported the association between upregulation of PD-L1 and poor survival in patients with HCC (8,42). Several previous studies have reported different mechanisms involved in the regulation of PD-L1 expression in cancer cells (19,43-45). However, the mechanism of PD-L1 expression in HCC still remains unclear (46).

In previous years, previous studies have demonstrated cytokine-induced EMT, in particular TNF- $\alpha$ , TGF- $\beta$  and interferon- $\gamma$  are responsible for elevating the expression of PD-L1 in cancer, such as non-small cell lung carcinoma and breast cancer (17,19,21). The present finding that TNF- $\alpha$  is involved in the upregulation of PD-L1 is consistent with these studies. Another previous study demonstrated that PD-L1 was upregulated in EMT positive human esophageal tumor samples compared with the EMT negative samples (23). The

relationship between EMT and PD-L1 was further examined in human breast cancer cells by Noman *et al* (20). This previous study identified increased expression of PD-L1 in cells having undergone EMT by EMT-transcription factors (zinc finger E-box binding homeobox 1, microRNA 200 or Snail) and PD-L1 rendered EMT-activated cells resistant to cytotoxic T-lymphocytes-mediated lysis (20). In patients with HCC, our group previously identified an association between PD-L1 expression and EMT status (8). High PD-L1 expression was closely associated with high expression of the mesenchymal marker Vimentin and low expression of epithelial marker E-cadherin (8). However, to the best of the authors' knowledge, no studies have reported the association between EMT and other immune checkpoint in HCC.

PD-L2 is a second ligand that binds to PD-1 to prevent cytokine production, cell adhesion and T-cell proliferation (47). Our group and other researchers have previously reported that PD-L2 expression is associated with poor survival and recurrence of HCC in patients (8,48). A previous meta-analysis study by Yang *et al* (49) identified that upregulation of PD-L2 predicted poor OS in HCC.

CD73 is also reported to be a novel prognostic biomarker for HCC (8,50,51). However, to the best of the authors' knowledge, the regulation of CD73 expression has not been studied in the context of EMT, and the present study is the first to report that TNF- $\alpha$ -induced EMT regulates CD73 expression. In a recent triple-negative breast cancer study by Qiao *et al* (52), an anti-CD73 antibody was demonstrated to inhibit lung metastasis *in vivo*.

B7-H3 is mostly upregulated in several cancer types, including liver, breast, bladder, colorectal, cervical, glioma, esophageal and gastric cancer (53). Previous studies have also demonstrated that dysregulation of B7-H3 in HCC results in impaired T-lymphocyte function, thus leading to poor prognosis and recurrence (54,55). In muscle-invasive bladder cancer, high expression of B7-H3 was associated with a poor clinicopathological status and poor prognosis (56). Most notably, B7-H3 is known to promote EMT by repressing E-cadherin expression, and upregulating Vimentin and N-cadherin expression in colorectal cancer (57). To the best of

the authors' knowledge, the present study is the first to report the regulation of B7-H3 expression by EMT in HCC.

Further mechanistic and functional studies are necessary to examine the molecular biology of immune checkpoints and its precise role in EMT in HCC. However, given the association between EMT, TNF- $\alpha$  and immune checkpoint in HCC, it is conceivable that combining EMT or TNF- $\alpha$  inhibitors with ICIs in patients with HCC may be a future therapeutic approach for the management of HCC.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

RS, KRB, DHGC and AJ designed the study, performed data acquisition, analysis and interpretation, and wrote the manuscript. RS, AJ, KRB and DHGC critically revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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