

## Hepatoma cells up-regulate expression of programmed cell death-1 on T cells

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### Abstract

**AIM:** To investigate the effect of hepatoma cells on up-regulation of programmed cell death-1 (PD-1), and the function of PD-1 on T cells.

**METHODS:** HepG2 or HepG2.2.1.5 cells were co-cultured with a lymphoma cell line-Jurkat cells. PD-1 expression was detected by flow cytometry. IL-2, INF- $\gamma$  and IL-10 in culture supernatant were detected by enzyme-linked immunosorbent assay (ELISA). Cytotoxic action of T cells was determined by MTT reduction assay-direct mononuclear cell cytotoxicity assay.

**RESULTS:** The PD-1 expression on Jurkat cells increased by 16.17%  $\pm$  2.5% and 17.43%  $\pm$  2.2% after HepG2 or HepG2.2.1.5 cells were co-cultured for 48 h. The levels of IL-2, INF- $\gamma$  and IL-10 in the culture supernatant were 202.9  $\pm$  53.0 pg/mL, 88.6  $\pm$  4.6 pg/mL and 63.7  $\pm$  13.4 pg/mL respectively, which were significantly higher than those (102.9  $\pm$  53 pg/mL, 39.3  $\pm$  4.2 pg/mL, and 34.6  $\pm$  13.7 pg/mL) in the control group ( $P < 0.05$ ). The OD value for MTT assay in the blocking group (0.29  $\pm$  0.06) was significantly higher than that (0.19  $\pm$  0.09) in the control group ( $P < 0.05$ ).

**CONCLUSION:** PD-1 expression on Jurkat cells is up-regulated by hepatoma cells, cytokines and cytotoxic action are elevated after PD-1/PD-L1 is blocked.

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**Key words:** Hepatoma cell; Programmed cell death-1; Protein expression; T cell function; Cytokine

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### INTRODUCTION

Programmed cell death-1 (PD-1), a member of the CD28 family, was first isolated from T cell hybridoma by subtractive hybridization in 1992<sup>[1]</sup>, and is expressed on activated T and B cells<sup>[2]</sup>. PD-1 has been recently found to play a role in immunity regulation as an inhibitory co-signaling molecule<sup>[3]</sup>. Upon its ligands (PD-L1 and PD-L2) are ligated, PD-1 decreases T cell receptor (TCR)-mediated proliferation, cytokine production and cytolytic activity<sup>[4-7]</sup>. Barber *et al*<sup>[8]</sup> showed that up-regulation of PD-1 expression on T cells is associated with the exhaustion of T cells in lymphocytic choriomeningitis virus (LCMV) infection. The number of effective T cells increases and their function markedly improves when the interaction of PD-1 and PD-L is blocked. It was reported that PD-1 also plays a significant role in some autoimmune diseases<sup>[9-11]</sup> and viral infectious diseases, especially in chronic infectious diseases caused by human immunodeficiency virus (HIV)<sup>[12-14]</sup>, hepatitis C virus (HCV)<sup>[15,16]</sup> and hepatitis B virus (HBV)<sup>[17-19]</sup>. There is evidence that PD-1 suppresses immune activation in PD-1-deficient mice with autoimmune disease<sup>[20]</sup>. Single-nucleotide polymorphisms at the PD-1 locus have also been identified in patients with autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes<sup>[21]</sup>.

Recent evidence from studies on tumors shows that the PD-1/PD-L pathway might play a critical role in tumor immunity evasion<sup>[22-24]</sup>. PD-1 is up-regulated on tumor specific T cells and PD-L is up-regulated in tumor tissue<sup>[25-27]</sup>. As a result, the function of tumor specific T cells is suppressed, leading to the immune escaping of tumor cells, and the level of mRNA and PD-L1

protein can be detected in tumor tissues<sup>[25-27]</sup>. In addition, PD-1/PD-L interaction promotes apoptosis of T cells, induces clearance of specific T cells, and ultimately inhibits the anti-tumor immunity response<sup>[27]</sup>. Blocking the interaction of PD-1/PD-L with anti-PD-1 antibody partially recovers the function of tumor specific T cells.

It was reported that the expression of PD-L can be induced by virus and cytokines such as IFN- $\alpha$  and INF- $\gamma$ <sup>[28]</sup>. However, no evidence for the induced expression of PD-1 is available. Liver, an important immune organ, plays a critical role in immune regulation although it is prone to induce immune tolerance in many cases. The present study was designed to investigate whether hepatoma cells (HepG2 and HepG2.2.1.5) induce expression of PD-1 in T cells. The functional role of PD-1/PD-L interaction was also studied.

## MATERIALS AND METHODS

### Cell culture

Jurkat cells were cultured in RPMI 1640 medium (Gibco, USA), supplemented with 10% fetal calf serum, 300  $\mu$ g/mL glutamine, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. HepG2.2.1.5 cells or HepG2 cells provide by Mountsinai Medical Center were cultured in complete DMEM (Gibco, USA) containing 380  $\mu$ g/L G418.

### Co-culture system

HepG2 and HepG2.2.1.5 cells were cultured in 6-well plates ( $5 \times 10^5$  cells/well) for 24 h and Jurkat cells ( $5 \times 10^5$  cells/well) were added and co-cultured for 48 h. The suspended cells were collected for analysis of PD-1. Jurkat cells were cultured solitarily for 48 h as controls.

### Analysis of PD-1 expression

Cell surface expression of PD-1 was detected by flow cytometry after incubated with allophycocyanin (APC)-conjugated anti-PD-1 antibodies (eBioscience). Cells were collected and suspended in PBS containing 1% fetal calf serum (FCS). The cell density was adjusted to  $1 \times 10^6$  cells/vial. After incubated with anti-PD-1 antibodies or matching isotype controls at 37°C for 30 min, cells were washed with PBS and fixed in 2% paraformaldehyde for analysis. A total of 20000 gated cells were analyzed on Becton Dickinson FACS (Becton Dickinson, USA) using the CELLQuest™ software.

### Blockade of PD-1/PD-L1 interaction

Antibody against human PD-L1 (eBioscience) was used to block PD-L1. Jurkat cells were activated with phytohemagglutinin (PHA) (2  $\mu$ g/mL, Sigma, USA) and co-cultured with HepG2 or HepG2.2.1.5 cells. Anti-PD-L1 antibodies (25  $\mu$ g/mL) were added into the culture to block the interaction of PD-1 and PD-L1 for 48 h. Mouse IgG, as a control antibody, was used in the control group. After cultured for 48 h, the supernatants of co-cultures were collected and stored at -80°C.

### Analysis of cytokine secretion

**Table 1** Up-regulated expression of PD-1 on T cells ( $n = 24$ , mean  $\pm$  SD)

Groups	PD-1(%)	
Controls	0.70 $\pm$ 0.03	
Co-cultured	HepG2	16.17 $\pm$ 2.5 <sup>a</sup>
	HepG2.2.1.5	17.43 $\pm$ 2.2 <sup>b</sup>

<sup>a</sup> $P = 0.000$ , <sup>b</sup> $P = 0.000$  vs control group.

Levels of IL-2, IL-10 and INF- $\gamma$  in the supernatants were measured by ELISA (Ucytech, Netherlands) following its manufacturer's instructions.

### Analysis of cytolytic activity

Cytolytic activity of Jurkat cells was detected by MTT assay. After co-cultured for 48 h, Jurkat cells were isolated and MTT was added to incubate HepG2.2.1.5 cells for 4 h, and bleached with DMSO. Finally, the cytolytic effect of Jurkat cells on HepG2.2.1.5 cells was analyzed on Bio-Rad 450 (USA).

### Statistical analysis

Results were expressed as mean  $\pm$  SD or percentage. Comparison between groups was made using Student's unpaired *t*-test.  $P < 0.05$  was considered statistically significant. All analyses were performed using SPSS 13.0 for Windows.

## RESULTS

### Enhancement of PD-1 expression on Jurkat cells

PD-1 expression on Jurkat cells was determined by FACS analysis at 48 h after co-cultured with HepG2 or HepG2.2.1.5 cells. Jurkat cells were also cultured solitarily as controls. The expression of PD-1 was induced on Jurkat cells after co-culture with HepG2 or HepG2.2.1.5 cells for 48 h, which was significantly higher on Jurkat cells co-cultured with hepatoma cells than on controls ( $P = 0.000$ , Table 1).

### Function restoration of T cells

Supernatants were collected from the blocking and control groups. To investigate the influence of cytokine production after PD-1/PD-L1 was blocked, the levels of IL-2, IL-10 and INF- $\gamma$  were measured. After PD-L1 was blocked with specific antibodies, the levels of IL-2, IL-10 and INF- $\gamma$  were much higher in the blocking group than in the control group ( $P = 0.000$ , Table 2).

Furthermore, the effect of the PD-1/PD-L1 pathway on cytolytic activity of T cells was also investigated by MTT assay. The *A* value ( $0.29 \pm 0.06$ ) in the blocking group was much higher than that ( $0.19 \pm 0.09$ ) in the control group ( $P = 0.000$ ).

## DISCUSSION

Activation of resting lymphocytes triggers expression of several products of the immunoglobulin superfamily of genes. These activation-induced antigens are involved in

**Table 2** Secretion of cytokines by T cells (pg/mL, *n* = 24, mean  $\pm$  SD)

Groups	IL-2	IFN- $\gamma$	IL-10
Control group	102.9 $\pm$ 53.0	39.3 $\pm$ 4.2	34.6 $\pm$ 13.7
Blocking group	202.9 $\pm$ 53.0 <sup>c</sup>	88.6 $\pm$ 4.6 <sup>d</sup>	63.7 $\pm$ 13.4 <sup>e</sup>

<sup>c</sup>*P* = 0.000, <sup>d</sup>*P* = 0.000, <sup>e</sup>*P* = 0.000 *vs* control group.

many physiological and pathological processes including cell proliferation (IL-2R), functional differentiation (CTLA-4), and apoptosis (Fas)<sup>[29,30]</sup>. The expression patterns of these antigens are cell-specific, and have different regulation functions in different cells. PD-1, a member of the CD28 family, which was isolated from apoptosis-induced T cell hybridoma in 1992<sup>[1]</sup>, is expressed on activated T and B cells<sup>[2]</sup>.

Agata *et al*<sup>[2]</sup> showed that PD-1 expresses on activated T and B cells. Anti-CD3 and concanavalin A (ConA) can stimulate its expression on thymocytes and T cells in spleen, and anti-IgM antibody can stimulate its expression on B cells in spleen. Vibhakar *et al*<sup>[31]</sup> also demonstrated that PD-1 mRNA and protein levels in Jurkat cells are up-regulated in a time-dependent manner during phorbol ester (TPA)-induced differentiation, indicating that lymphocyte activators can up-regulate PD-1 expression on lymphocytes. Since PHA is another T cell activating agent, the expression of PD-1 in T cells can be detected after stimulation of PHA. A time-dependent up-regulation of hPD-1 was also observed during PHA induction (data not shown), and was used as a stimulus of Jurkat cells in our blocking experiment.

It was reported that, as an inhibitory co-stimulating molecular, PD-1 plays a role in immune regulation and is associated with the exhaustion of effective T cells<sup>[32-34]</sup>. Barber *et al*<sup>[8]</sup> showed that, in chronic viral infection diseases, PD-1 is highly expressed on the exhausted LCMV-specific CD8 T cells and blocking the PD-1/PD-L1 interaction during the chronic phase of infection can efficiently reanimate the exhausted CD8 T cells and promote clearance of the persisting virus. In contrast, PD-1 expression is transiently induced and declines quickly to its basal level in acute LCMV-Armstrong infection, thus promoting studies on other diseases associated with immune. Up-regulation of PD-1 expression on effective T cells leads to suppression of immune, which might be the underlying mechanism of immune evasion. PD-L1/PD-L2 expression in a variety of tumor cells has been detected in human tumors<sup>[35,25-27]</sup>, while PD-1 over-expression on tumor specific T cells has also been observed<sup>[23]</sup>. Interaction of PD-1/PD-L1 promotes apoptosis of T cells, inhibits anti-tumor immune response of T cells, and stimulates growth of tumors<sup>[27]</sup>. Obstructing the interaction of PD-1/PD-L1 enhances the function of T cells, hampers development of tumors<sup>[22,23,26]</sup>. In the present study, tumor cells induced expression of PD-1 on T cells. After co-cultured with hepatoma cells, PD-1 was expressed on T cells, but not expressed on Jurkat cells after cultured for 48 h solitarily. PD-1 was expressed on T cells after Jurkat

cells were co-cultured with the supernatant of hepatoma cells (data not shown), suggesting that hepatoma cells can up-regulate the expression of PD-1.

These findings lead to the clinical use of PD-1 blockers in the treatment of tumors. In chronic infectious diseases, virus can induce the expression of PD-1 on T cell. However, the precise mechanism PD-1 blockers still remains unclear. We observed the effect of tumor cells on PD-1 expression in T cells. Jurkat cells, a kind of CD4<sup>+</sup> T cells, can be used as target cells co-cultured with hepatoma cells. FACS analysis showed that tumor cells could induce PD-1 expression on T cells. In this study, HepG2.2.1.5 cells transferring HBV genome and HepG2 cells not transferring HBV genome could induce PD-1 expression, suggesting that HBV has no effect on PD-1 expression on T cells.

In addition, the function of PD-1 on T cells was also observed. Anti-PD-L1 antibody was used to block the interaction of PD-1 and PD-L1. PD-1 was induced by PHA, and PD-L1 was expressed in HepG2.2.1.5 cells identified by FACS (data not shown). Jurkat cells after activated by PHA were co-cultured with HepG2.2.1.5 cells. Antibodies against human PD-L1 were added into the co-culture system as a blocking group, while mouse IgG was added as a control group. The levels of cytokines including IL-2, INF- $\gamma$  and IL-10 in culture supernatant and the cytolytic activity of T cells were detected, which were significantly elevated in blocking group compared to the control group, suggesting that both Th1 and Th2 have immune responses can be restored and PD-1/PD-L1 that negatively regulates the immune reaction by blocking the PD-1/PD-L pathway can recover the function of T cells, which introduces a new theory of tumor immune evasion. This new mechanism of tumor immunology might provide a novel target for therapy.

## COMMENTS

### Background

Programmed cell death-1 (PD-1), originally isolated from apoptotic T cells, is a 55-kDa transmembrane protein with an extracellular IgV-like domain and a 97-amino acid cytoplasmic tail containing an immunotyrosine inhibitory motif (ITIM) and an immunotyrosine switch motif (ITSM). PD-1 has two ligands: PD-L1 and PD-L2, which are members of the CD28/B7 superfamily. The expressions of CD28, CTLA-4, and ICOS are limited in T cells. PD-1 can be expressed on activated T, B and myeloid cells. The expression of PD-L1 has been detected in many organ tissues, such as heart, lung, pancreas, muscle, and placenta, including lymphocytes and non-lymphocytes. The expression of PD-L2 is restricted in DC and macrophages.

### Research frontiers

Recent findings suggest that PD-1/PD-L pathway plays a role in regulating tolerance and autoimmunity. The role of PD-1 and its ligands in regulating human autoimmune disease, infectious diseases and tumors has been investigated. Interaction of PD-1 and PD-L has been found to be important for controlling effective T cells. Significantly increased expression of PD-1 and PD-L1 in T and B macrophages/dendritic cells and tumor cells, associated with T-cell exhaustion and disease progression, immobilized auto-antibodies to PD-L, can stimulate T cell proliferation, cytokine production, and programmed cell death. The up-regulation of PD-L1 in tumors and PD-1 in T cells can lead to immune tolerance.

### Innovations and breakthroughs

The present study demonstrated the effect of hepatoma cells on up-regulating

the expression of PD-1 in T cells. In addition, the function of PD-1 in T cells was assessed, showing that the cytokine production and cytotoxicity of T cells can be elevated by blocking the interaction of PD-1 and PD-L1.

### Applications

The up-regulation of PD-1 in T cells by hepatoma cells has led to a new hypothesis that the PD-1 and PD-L pathway may be a means by which tumors evade T cell recognition. Manipulation of the PD-1 pathway can enhance immune response, and may become a novel strategy for the treatment of tumors.

### Terminology

MTT is a direct mono-nuclear cell direct cytotoxicity assay used to detect the cytotoxicity of T cells. PHA is a T cell stimulus that can activate T cells. G418, a kind of antibiotics, is used in selective cell culture medium. G418 must be added into culture medium of HepG2.2.1.5 cells to support the selective survival of HepG2.2.1.5 cells.

### Peer review

This is a very interesting study, showing that HCC-induced modulation of PD-1 expression in T-cells might contribute to immune evasion. This concept might hold its promise for new therapeutic interventions. The experiments support the authors' claim.

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