

Original

**Anti-TIM-3 Antibody Prevents Lymphocyte Apoptosis
and Enhances Dendritic Cell Cancer Therapy**

Eiichi HAYASHI, Junichi EGUCHI*, Masashi SAKAKI,
Hiroyoshi DOI, Risa OMORI, Atsushi KAJIWARA
and Hitoshi YOSHIDA

Abstract : Currently, there is a strong unmet need for a new intervention therapy for hepatocellular carcinoma (HCC). One candidate therapy uses dendritic cells (DCs), which are professional antigen-presenting cells that are characterized by their potent ability to elicit immune responses to foreign antigens. DCs may be attractive adjuvant agents for cancer therapy but the effect of DCs therapy is restricted because of the immunosuppressive nature of the tumor microenvironment. T-cell immunoglobulin and mucin protein-3 (TIM-3) is a marker of this immunosuppressive tumor environment. Interaction of TIM-3 with its ligand, galectin 9, triggers cell death in activated T cells. In this study, we evaluated the antitumor effects of DC vaccine therapy in combination with TIM-3 blockade in a murine HCC system. In an animal cancer prevention model, BALB/c mice were immunized with DCs with or without anti-TIM-3 antibody before challenging with HCC tumor cells (BNL). In an animal cancer therapeutic model, BALB/c mice were inoculated with DCs with or without anti-TIM-3 antibody 10 days after injection with BNL cells. The mechanism of this combination therapy was investigated using immunohistologic staining of the treated tumors and flow cytometry of lymphocytes. DC and anti-TIM-3 antibody combination treatment prevented tumor development to a greater extent than DCs alone. In the therapeutic model, the outgrowth of the established tumors was significantly reduced in mice treated with the combination of DCs and anti-TIM-3 antibody. Immunohistological analyses of the therapeutic model showed marked infiltration of CD4⁺ cells and CD8⁺ T-cells in the established BNL tumors of mice treated with both DCs and anti-TIM-3 antibody. Anti-TIM-3 antibody treatment reduces lymphocyte apoptosis and enhances the antitumor effect of DC therapy in a murine HCC model.

Key words : dendritic cells, T-cell immunoglobulin and mucin protein-3, hepatocellular carcinoma, immunotherapy

Introduction

Approximately 500,000 new cases of hepatocellular carcinoma (HCC) are diagnosed per annum worldwide. Vaccination against hepatitis B virus (HBV) and the use of direct acting

Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.

* To whom corresponding should be addressed.

antiviral agents (DAAs) therapy against hepatitis C virus (HCV) is expected to reduce the number of HCC patients in the future. Nevertheless, the incidence of HCC is still increasing in Asia and Africa because of the prevalence of these viral infections in those regions. Progress in the development of treatments for HCC has resulted in an improved prognosis for HCC patients. Unfortunately HCC is usually associated with cirrhosis and often recurs even after effective treatment of the tumors in the remaining part of the cirrhotic liver. Thus, there is a strong unmet need for the development of a new and effective intervention therapy that suppresses HCC occurrence or recurrence and has fewer side effects. Initial clinical trials have been performed using immunotherapy and these indicate that it may be applicable to the clinical treatment of HCC.

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that are characterized by their potent ability to activate and stimulate naïve T-lymphocytes *in vivo*¹⁾. Immature DCs take up and process antigens present in the lymph node or in peripheral organs. Antigen-loaded DCs move to draining lymph nodes where DCs present the antigens to naïve T-cells and enhance their proliferation and differentiation into effector T-cells²⁾. The co-localization of DCs and tumor cells may generate APCs capable of stimulating tumor-reactive T cells *in vivo*. The histologic infiltration of DCs into primary tumors is associated with prolonged patient survival and reduces the incidence of metastatic disease in patients with bladder, lung, esophageal, gastric, and nasopharyngeal carcinoma³⁻⁸⁾. A number of clinical studies have been performed that indicate that DCs are attractive adjuvants as well as therapeutic agents for cancer therapy in murine models⁹⁻¹²⁾. However, DC therapy is not sufficient to cure HCC because patients with these tumors have a suppressed immune system.

T-cell immunoglobulin and mucin protein-3 (TIM-3) is expressed on IFN- γ -secreting T helper 1 (Th1) cells as well as on DCs, monocytes, CD8⁺ T-cells and other lymphocyte subsets¹³⁾. Interaction of TIM-3 with its ligand, galectin-9, triggers cell death in TIM-3⁺ T-cells, which implicates TIM-3 in negatively regulating the Th1 response¹⁴⁾. The blockade of TIM-3 activity results in an increase in IFN- γ -secreting cells in the immunosuppressive tumor microenvironment^{15, 16)}. TIM-3 is also expressed on macrophages and monocytes and has been implicated in both the phagocytosis of apoptotic cells and cross-presentation¹⁷⁾. Other immune checkpoint molecules including cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed cell death 1 (PD-1) are currently at the forefront of cancer immunotherapy. Anti-CTLA-4 antibody has been approved for the treatment of advanced melanoma, and anti-PD-1 antibody has already shown promise in early clinical trials¹⁸⁻²¹⁾.

We have previously reported that the combination of IFN- α gene-transduced tumor-based vaccination therapy in combination with anti-PD-1 antibody suppresses the outgrowth of established tumors in a murine colorectal cancer model²²⁾. Now we propose that an anti-TIM-3 antibody can enhance the effect of existing immunotherapy.

In this study, the antitumor effects of combined DC vaccine therapy with TIM-3 blockade were evaluated in a murine HCC system as a preliminary investigation prior to clinical studies. We show that the outgrowth of the established tumors is significantly suppressed when tumor-

bearing mice are injected with DCs and anti-TIM-3 antibody. Furthermore, we performed immunohistologic staining of the tumors and flow cytometry lymphocytes to explore the mechanism of the antitumor effects induced by this combination therapy.

Materials and methods

Mice

Female 6-week-old BALB/c mice were purchased from Sankyo Lab Service (Tokyo, Japan) for experimental use between 8 and 12 weeks of age. The mice were maintained in an animal care facility at Showa University. The ethical committee for Animal Experiments of Showa University approved this study (permission #2011-1111).

Cell lines, culture medium and reagents

The BNL murine HCC cell line is of BALB/c origin and was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU penicillin and 100 µg/ml streptomycin in a humidified incubator with 5% CO₂ in air at 37°C. All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA). Phenotypically mature DCs and irradiated 100 Gy BNL cells were co-incubated to generate immunized DCs. Anti-TIM-3 antibody was purchased from eBioscience (San Diego, CA, USA).

Preparation of DCs and incubation with tumor cells

Bone marrow cells were isolated from BALB/c mice and DCs were generated by treatment of these cells with 10 ng/ml murine Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and 10 ng/ml Interleukin-4 (IL-4) (R&D Systems, Inc., Minneapolis, MN, USA). DCs were pre-incubated with BNL cells that had been previously γ -irradiated (100 Gy) at a DC to tumor cell ratio of 10:1²³⁾.

Prevention model

BALB/c mice were immunized before tumor injection with 200 µg of anti-TIM-3 antibody, or with 1×10^6 DCs with or without 200 µg of anti-TIM-3 antibody in the right flank on days 1, 8 and 15. PBS and immunized DCs were injected subcutaneously and anti-TIM-3 antibody was injected intraperitoneally²⁴⁾. On day 21, the mice were challenged with 1×10^5 BNL cells into the contra-lateral (left) flank and they were observed for 60 days. Each experiment involved five mice per group and was performed twice.

Therapeutic models

In order to evaluate the potential to treat established tumors, we measured the size of established BNL tumors of mice treated with DCs with or without anti-TIM-3 antibody. First, BALB/c mice were injected subcutaneously with 1×10^5 cell BNL in left flank. On days 7, 9 and 11 after the BNL inoculation, the mice were injected with 200 µl of PBS or 1×10^6 DCs or 200 µg of anti-TIM-3 antibody into contra-lateral (right) flank. Each experiment involved five

mice per group. Tumor size was measured twice a week using vernier calipers. Experiments for this model were performed twice.

Immunohistologic analysis

BALB/c mice were injected subcutaneously in the left flank with 5×10^5 BNL cells and after 14, 17 and 21 days, the mice were inoculated into the contra-lateral (right) flank with 200 μ l of PBS or 1×10^6 immunized DCs with or without 200 μ g of anti-TIM-3 antibody. Tumor tissues were harvested seven days after the last inoculation, and immediately embedded in optimal cutting temperature compound and frozen. Serial 5-mm sections were exposed to anti-CD4, anti-CD8a, anti-CD11c and anti-Gr-1 antibodies and anti-TIM-3 antibody (Nippon Becton Dickinson, Tokyo, Japan). Rat IgG2a (Nippon Becton Dickinson) was used as a control antibody. Immunostaining was completed with the PLP Solution set (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Immunoreactive cells were observed using light microscopy ($\times 400$) and counted in 10 fields. Each experiment involved two mice per group.

Flow cytometry of lymphocytes from immunized mice stimulated with DCs or anti-TIM-3 antibody

Mice were injected with 5×10^5 DCs as a vaccine three times on days 1, 8 and 14, and challenged with 5×10^5 BNL cells on day 21. Splenocytes were harvested seven days after the tumor inoculation and cultured *in vitro*. A total of 2×10^6 splenocytes were co-cultured with 5×10^5 DCs with or without 100 μ g of anti-TIM-3 antibody. The cultured splenocytes were re-stimulated with 30 IU/ml recombinant murine IL-2 (Becton Dickinson) after seven days of culture and then incubated for an additional seven days. The immunogenic effects of DCs and anti-TIM-3 antibody were analysed by flow cytometry using a FACsCalibur (Nippon Becton Dickinson).

CD4⁺ T-cells and CD8⁺ T-cells were sorted and isolated using MACs beads (Miltenyi Biotec, Bergisch Gladbach, Germany) with either CD4 or CD8 beads. The isolated cells were stained with fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated anti-H-2K^b, CD4, CD8, and caspase-8 monoclonal antibodies (Medical & Biological Laboratories Co, Ltd, Nagoya, Japan). 7-Amino-Actinomycin D (7-AAD) (Nippon Becton Dickinson), which is a fluorescent dye that intercalates with cellular DNA and undergoes a spectral shift upon association, was used to detect dead cells.

Statistical analyses

The comparative growth of established tumors and T-cell responses were assessed using Student's *t*-tests for two samples with unequal variances. Differences between groups were considered significant when the *P* value was less than 0.05.

Results

Combined treatment with DC vaccine and anti-TIM-3 antibody prevents development of BNL tumors

BALB/c mice received three inoculations of DCs with or without anti-TIM-3 antibody. The

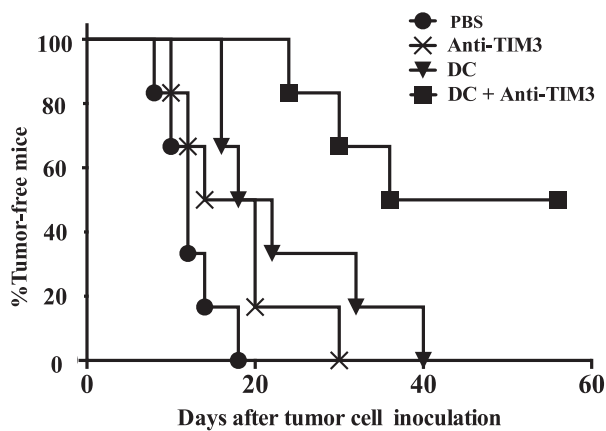


Fig. 1. Treatment of mice with anti-TIM3 antibody and a DC vaccine prevents the development of BNL tumors. Mice were injected three times with DCs with or without anti-TIM-3 antibody. Seven days after the last treatment, the mice were inoculated with BNL tumor cells, and observed for 60 days to determine the number of tumor-free mice (Control vs. DCs + anti-TIM-3: $P = 0.0014$, anti-TIM-3 vs. DCs + anti-TIM-3: $P = 0.0046$, DCs vs. DCs + anti-TIM-3: $P = 0.0305$).

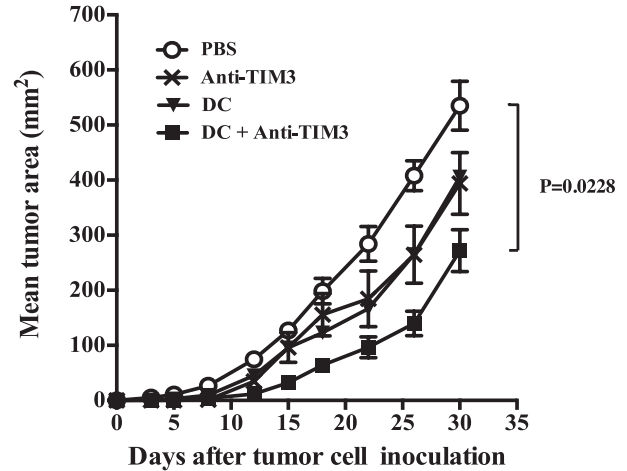


Fig. 2. Treatment of mice with DC vaccine and the anti-TIM-3 antibody suppressed the growth of established BNL tumors *in vivo*. Mice were inoculated with BNL in the flank on day 0. Established BNL tumors were treated three times with DCs alone, anti-TIM-3 antibody alone, or the combination in the contralateral flank every two days starting seven days after BNL cells injection.

mice were then challenged with an injection of BNL tumor cells on day 21. The duration for tumor establishment in BALB/c mice as a palpable tissue was measured over 60 days.

The mouse combination treatment group had the longest duration of tumor establishment compared to other treatment groups (Control vs. DCs + anti-TIM-3: $P = 0.0014$; anti-TIM-3 vs. DCs + anti-TIM-3: $P = 0.0046$; DCs vs. DCs + anti-TIM-3: $P = 0.0305$; Fig. 1). Therefore, the anti-TIM-3 with the DC vaccine had a preventive effect on tumor development. Tumor challenge was rejected for more than 60 days in half of the 10 mice treated with combined DCs and anti-TIM-3 antibody. A palpable tumor was observed in all of the mice tested in other groups.

Therapeutic inoculations with combined DCs and anti-TIM-3 antibody suppressed the growth of established BNL tumors in vivo

Mice bearing established BNL tumors were treated by an injection of DCs alone, or anti-TIM-3 antibody alone or by a combination of both. Seven days after the mice were injected with BNL cells they were injected with the DCs with or without anti-TIM-3 antibody in the opposite flank at three day intervals. We observed therapeutic effects by measuring the mean tumor area after tumor inoculation in all five mice in each treatment group. The mean area of established tumors in mice treated with both DCs and anti-TIM-3 antibody was significantly decreased compared with the area of the control group or single treatment groups (DCs + anti-TIM-3 antibody vs. DCs on day 30 tumor size, $P = 0.0228$; Fig. 2).

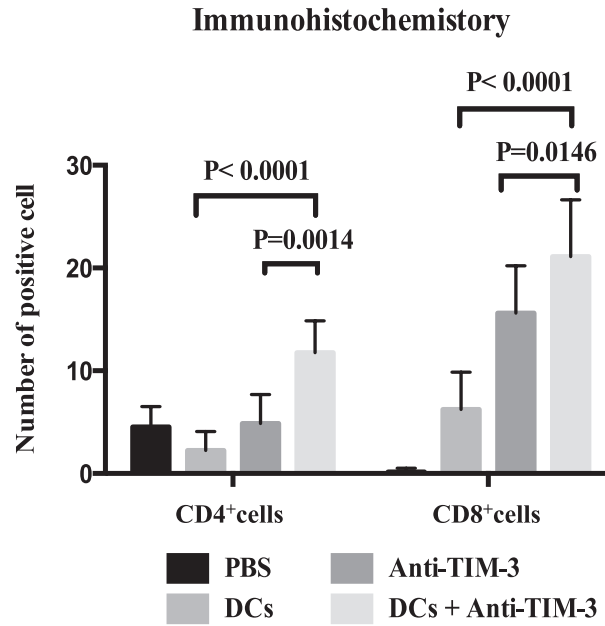


Fig. 3. CD4⁺ and CD8⁺ T-cells markedly infiltrated into the established BNL tumors of mice inoculated with the combination of DCs and anti-TIM-3 antibody. BALB/c mice were injected subcutaneously three times with DCs and anti-TIM-3 antibody either alone or in combination into the contralateral flank of the established BNL tumor. Tumor tissues were harvested 10 days after therapeutic inoculation and stained with anti-CD4 and anti-CD8 antibodies. Immunoreactive cells were observed using light microscopy ($\times 400$) and counted in 10 fields.

Combined DCs and anti-TIM-3 antibody treatment induced the infiltration of CD4⁺ and CD8⁺ cells in established BNL tumors

We performed immunohistochemical staining of BNL tumor tissues from treated mice to investigate the antitumor mechanisms induced by the DC and anti-TIM-3 therapy. There was marked infiltration of the tumors by both CD4⁺ cells and CD8⁺ cells in the single anti-TIM-3 antibody treatment group compared to the group treated with DCs alone (Fig. 3). In addition, there was no significant change in the infiltration of DC marker CD11c⁺ cells and granulocyte marker Gr-1⁺ cells in the established tumors of every group (data not shown).

Anti-TIM-3 antibody prevents apoptosis of lymphocytes from mice injected with BNL cells

Flow cytometry was performed to investigate the immunogenic effects of DC treatment and anti-TIM-3 treatment. Mice were injected with 1×10^6 tumor antigen-loaded DCs on the days 1, 8 and 17. On day 25, mice were inoculated with 1×10^5 BNL cells; they were sacrificed on day 32 and their spleens was harvested. After 14 days of culture, MACs beads were used to isolate to CD4⁺ and CD8⁺ T-cell populations from the splenocytes and these were analyzed by flow cytometry. Caspase-8 is a cell marker that is used to quantitatively determine the percent-

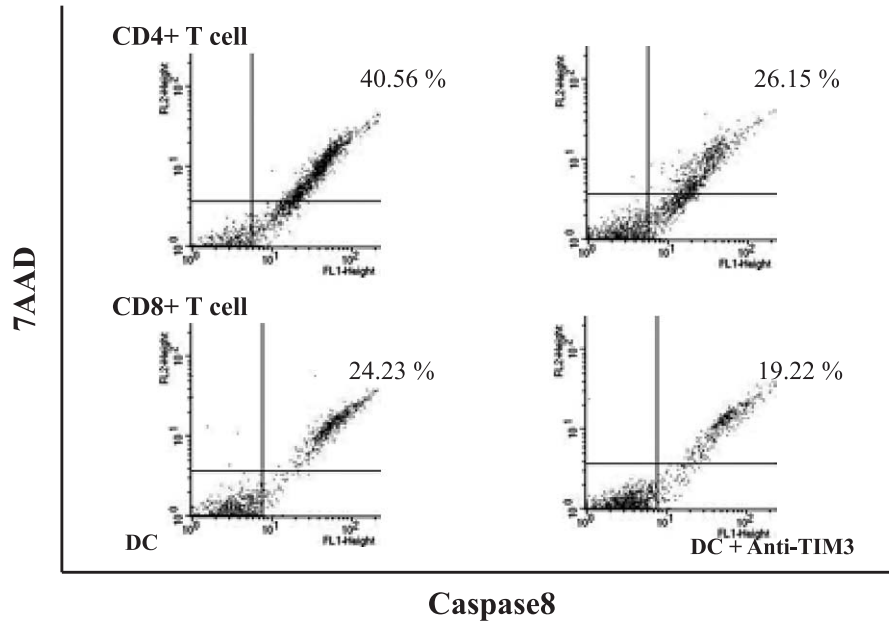


Fig. 4. Blockade of TIM-3 reduced the number of apoptotic lymphocytes in mice immunized with BNL. Splenocytes from mice immunized with irradiated BNL were stimulated with DCs or anti-TIM-3 antibody. After seven days of culture, splenocytes were isolated to CD4⁺ and CD8⁺ T cells and stained with the 7AAD and fluorescein isothiocyanate (FITC)-conjugated anti-caspase-8. Numbers in each histogram indicate the percentage of 7AAD⁺ / caspase-8⁺ cells in the total CD4⁺ or CD8⁺ population.

age of apoptotic cells within a population, whereas 7-AAD is a marker of the cell death. Viable cells are caspase-8⁺ and 7-AAD⁻; cells that are in early apoptosis are caspase-8⁺ and 7-AAD⁻; and cells that are in late apoptosis or are dead are both caspase-8⁺ and 7-AAD⁺. Treatment with anti-TIM-3 antibody decreased the population of caspase-8⁺, 7-AAD⁺ T-cell subsets (Fig. 4). These data indicate that addition of anti-TIM-3 antibody reduces the level of apoptosis in both CD4⁺ and CD8⁺ T-cell populations.

Discussion

DC therapy has been used previously for patients with advanced tumors such as melanoma and renal cancer but the outcomes have been unsatisfactory most likely as a result of the immunosuppressive environment in advanced cancer patients. We investigated TIM-3, which has been identified as a marker of immune suppressor of T-cells, with the aim of improving treatment in clinical applications.

TIM-3 was originally identified by its selective expression on IFN- γ -secreting Th1 and Tc1 cells. The interaction of TIM-3 with its ligand, galectin-9, triggers cell death in TIM-3⁺ T-cells¹⁶⁾. A recent study in patients infected with HIV showed that expression of the immune regulator TIM-3 is induced on exhausted CD8⁺ T-cells²⁵⁾. TIM-3 can function as a negative regulator of T-cell responses so that blocking TIM-3 can be effective against established tumors if TIM-3⁺ PD-1⁺ T cells are the predominant T-cell subset among tumor infiltrating lymphocytes (TILs) at

the commencement of treatment²⁶⁾.

This study was a preliminary investigation of combination therapy prior to clinical studies. The anti-tumor effects of DC vaccine therapy in combination with TIM-3 blockade were evaluated in a murine HCC system. Firstly, we examined the preventive effect of anti-TIM3 antibody and DC vaccine on tumor development. The mice received three vaccinations with DCs with or without anti-TIM-3 antibody prior to injection with tumor cells. The mice in the combination treatment group showed the longest duration for tumor establishment compared to other treatment groups. Secondly, we examined the therapeutic effect of DCs and anti-TIM-3 antibody in an established tumor model. We treated tumor-bearing mice with DCs and anti-TIM-3 antibody, and observed a significant suppression of outgrowth of the established tumors in the combination treatment group compared to DCs single treatment group. These results suggest that DC and anti-TIM-3 antibody combination treatment has an additive antitumor effect in both the prevention and therapeutic models.

Immunohistologic analyses of the established tumors in mice treated with anti-TIM-3 antibody showed marked infiltration of both CD4⁺ and CD8⁺ T-cells, compared with the control group and the group treated with DCs alone. These data support the results of a previous study that blockade of TIM-3 signaling pathways reverses T cell exhaustion and restores antitumor immunity²⁷⁾. We also demonstrated that blockade of TIM-3 reduced the level of apoptosis in lymphocytes *in vitro*. These observations suggest that the TIM-3 blockade prevented lymphocyte apoptosis and maintained the survival and infiltration of tumor-specific T-cells in the local tumor environment.

In conclusion, our findings suggest that the combination of DC immunotherapy and TIM-3 blockade therapy has the potential to induce potent immune responses. Therefore, this combination therapy should be investigated further as a possible candidate for clinical trials of cancer vaccines.

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Conflict of interest disclosure

The authors have declared no conflict of interest.

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