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Animal and In Vitro Models

Tumor-derived CD4 + CD25 + regulatory T cells inhibit dendritic cells function by CTLA-4

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ABSTRACT

Purpose: CD4 + CD25 + regulatory T cells (Tregs) play an important role in anti-tumor immune responses. Poor prognosis and declining survival rates have intimate connection with high Treg expression in cancer patients. Cytotoxic T Lymphocyte-associated protein (CTLA-4) is one of the most prominent molecules on Treg. In our previous research, we have demonstrated that HCC-derived Tregs can interfere with Dendritic cells (DCs) function and down-modulate CD80/CD86 on DCs in vitro in a cell-contact dependent way. However the mechanism of how HCC-derived Treg affect DC phenotype are not very clear. Therefore, we investigated the function of CTLA-4 in anti-tumor immune responses.
Materials and methods: We established BABL/C mouse with hepatocellular carcinoma model, and tumor-derived Tregs were purified by magnetic cell sorting using mouse CD4 + CD25 + regulatory T cell isolation kit. Splenic DCs were enriched using CD11c-conjugated microbeads. Then splenic DCs co-cultured with tumor-derived Tregs and antibody-blocking experiments was performed.
Results: In our research, we found the down-modulation of CD80/CD86 on DCs was inhibited by blocking CTLA-4. HCC-derived Tregs down-modulated CD80/CD86 on DCs in a CTLA-4-dependent way. Blockade of CTLA-4 can lead to increase DC-mediated immunity.
Conclusion: CTLA-4 play a vital role in Treg-mediated immnue inhibition and this discovery can open up new ideas for the development of therapeutic strategies.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer worldwide [1]. Chronic viral hepatitis including hepatitis B virus or hepatitis C virus infection is a risk factor for HCC, making HCC prevention a major goal of antiviral therapy [2–4]. Treg-mediated suppression serves as a vital mechanism of negative regulation of immune-mediated inflammation and features prominently in autoimmune and autoinflammatory disorders, allergy, acute and chronic infections, cancer, and metabolic inflammation [5]. Many researches have proved the existence of Treg at tumor sites, indicating that Treg may induce local immune tolerance. The presence of infiltrating Tregs suppresses protective anti-tumor immune responses and their accumulation into the tumor microenvironment correlates with reduced survival [6]. Treg-derived from HCC patients have been proved to be more suppressive than Treg from healthy individuals [7]. Treg-mediated suppression can be important targets for immune intervention. Blockade of Treg-mediated immune suppression can enhance anti-tumor effects [8,9]. Combinations of monoclonal antibodies capable of modulating Treg functions synergistically enhance antitumor activity [10]. Cytotoxic T Lymphocyte-Associated Antigen 4 (CTLA-4) expressing on Tregs has been implicated in the function of Tregs. Li et al. found cryoablation and anti-CTLA-4 antibody in prostate cancer murine model induced anti-tumor immunological response [11]. There are many mechanisms have been proved for Treg-mediated suppression. In our previous research [12], increase frequency of Tregs in the peripheral blood and spleen was found in hepatocellular carcinoma mice. HCC-derived Treg could down-regulate the expression of costimulatory molecules CD80/CD86 on DCs by cell-to-cell contact and inhibit TNF-a and IL-12 production from DC. However the mechanism is not very clear. We still need deeper studies on the role of Treg in patients with hepatocellular carcinoma to provide new approaches for tumor immunotherapy. In our study, there is obvious tendency for cell cluster formation between tumor-derived Treg and DC. Treg-DC cluster formation may help Tregs to regulate the phenotype and function of DC. The down-modulation

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of CD80/CD86 is inhibited by blocking CTLA-4. IL-12 is one of important stimulators produced by DCs. When a high concentration of anti-CTLA-4 antibody is added in the Treg-DC group, the down-modulation of IL-12 is inhibited partially too. Our research illustrates that blockade of CTLA-4 by addition of anti-CTLA-4 mAbs can lead to increased DC-mediated immunity.

2. Material and methods

2.1. Tumor model

Male BALB/C mice (6–8 weeks old, purchased from the SLAC-CAS company,China) were bred under specific pathogen-free (SPF) conditions. Animal protocols were approved by the Experimental Animal Management Committee Of Wenzhou Medical University. All surgery was performed under chloralic hydras anesthesia, and all efforts were made to minimize suffering.

The H22 hepato-cellular carcinoma cells (China Center for Type Culture Collection, wuhan, CN) were cultured in RPMI 1640 medium and cells were inoculated to abdomen with 1×10^6 cells per mouse. After 8–9 days, cancerous ascites were extracted aseptically. Cells were collected and resuspended to a concentration of 1×10^6/ml for making model. The mice were randomly divided into the tumor group and control group. The mouse was celiac anesthesia. We did a longitudinal incision, exposed the liver, injected 0.01 ml cancer cell suspension (10^4 cells) to the liver and last closed enterocoelia. About 25 days later, the models were formed. When anatomy, we could see gray and nubbly carcinoma tissues at different sizes from 0.5 to 1.0 cm in diameters in livers. 4-μm thick sections were prepared and stained with haematoxylin and eosin by standard histological procedures (Fig. 1).

2.2. Reagents and materials

Mouse CD4 + CD25 + regulatory T cell isolation kit and mouse CD11c magnetic bead sorting kit (MACS, Miltenyi Biotec, Germany); Fluorescentlylabeled antibody: CD4-FITC, CD25-PE, CD11c-FITC, CD86-PE, CD80-PE and corresponding homotype antibody (eBioscience, California, USA); Mouse IL-12 ELISA kit (IBL, Hamburg, Germany); anti-CTLA4(R&D, Minnesota, USA); Anti-CD3 antibody (BD biosciences, New Jersey, USA); LPS(Sigma, Santa clara, CA, USA); FCS and RPMI1640 (Gibco, Grand Island, New York, USA); mouse H22 hepatocellular carcinoma cell lines (China Center for Type Culture Collection, wuhan, CN); Flow cytometry (BD biosciences, New Jersey, USA).

2.3. Cell isolation and sorting

Splenic single-cell suspension was prepared from normal mice. Then splenic DCs were enriched using CD11c-conjugated microbeads according to the manufacturers' instructions. The cells were positive for CD11c as assessed by flow cytometry. High purity of DCs were got finally (Fig. 2).Splenic single-cell suspension was prepared from HCC-bearing mice and then tumor-derived Tregs were purified by magnetic cell sorting using mouse CD4 + CD25 + regulatory T cell isolation kit according to the manufacturers' instructions. High purity of tumor-derived Treg or CD4 + CD25- Teff cells were gathered respectively (Fig. 2). Both T cells were stimulated separately with plate-bound anti-CD3 antibody (1 μg/ml) and IL-2 (10U/ml) at 5×10^5/ml in 24-well plates. Preactivated tumor-derived Tregs ($4–8 \times 10^5$/ml) or Teff cells ($4–8 \times 10^5$/ml) or a mix of two population at a 1:1 ratio were cultured with splenic DCs (DC-T cell ratio of 1:2) and then anti-CTLA-4 mAb (100 μg/ml) or control Ab were added. LPS was added(1 μg/ml)to stimulate DCs. Last, the co-cultures and the supernatants were collected respectively.

2.4. Flow cytometry and elisa

The co-cultures and the supernatants were collected respectively. The samples were stained with corresponding antibodies at 4 °C for 30 min and analyzed by flow cytometry. Isotype-matched antibodies were used as controls. IL-12 was assessed by ELISA using corresponding enzyme-linked immunosorbent assay kits according to the manufacturer' s instruction.

2.5. Statistical analysis

Data are expressed as mean ± sd. Statistical analysis was performed using Student's *t*-test with SPSS18.0 program (SPSS, Chicago). All P values <0.05 were considered statistically significant.

3. Results

3.1. Tregs form stable cluster with DCs

Pre-activated tumor-derived Tregs were co-cultured with splenic DCs in the presence of LPS, and typical cluster formation was observed (Fig. 3). Onishi et al. confirmed that Tregs form aggregates around Splenic DCs using immunofluorescence [13]. In our previous research(12), we have confirmed Tregs down-regulated the expression of costimulatory molecules CD80/CD86 on DCs by cell-to-cell contact. This indicated that Treg-DC cluster formation might enable Tregs to modulate phenotypic and functional characteristics of DCs. Tregs might diminish the up-regulation of costimulatory molecule expression on DC by formation stable cell cluster. Treg-DC cluster might constitute a physical barrier and interfere with the function of DC.

3.2. HCC-derived tregs down-regulate CD80/86 expression on splenic DCs depending on CTLA-4

Tregs could down-regulate CD80/86 expression on splenic DCs in our previous research, but by what mechanism for Treg mediating CD80/86 down-regulation was unkonwn. To further address the suppression mechanism induced by Tregs, we then analyzed the possible contribution of CTLA-4 and performed antibody-blocking experiments (Fig. 4). HCC-derived Treg either when co-cultured with DC separately or when co-cultured with DC and Teff simultaneously, reduced the expression of CD80/CD86. But when splenic DCs were co-cultured with HCC-derived Tregs in the presence of anti-CTLA-4 mAb, the CD80/86 expression was found not to down-regulate. To avoid cross-linking by anti-CTLA-4, control antibody was added to the cultures. The findings indicated that there exsited CTLA-4/B7 interaction. HCC-derived Tregs down-regulated CD80/86 expression in a CTLA-4 dependent manner and blockade of CTLA-4 with anti-CTLA-4 mAb could abrogate the Treg-mediated suppression.

3.3. CTLA-4 expression strengthen treg-mediated suppression

The production of pro-inflammatory and immunoregulatory cytokines by DCs is important for the generation of adaptive immune responses. IL-12 is one of important stimulator produced by DCs. LPS-induced mature DCs can secrete high level of IL-12 while Tregs down-regulate the production of IL-12 from DCs. But how CTLA-4 affect the cytokine secretion by DCs. To figure out this problem, we did antibody-blocking experiments. We added a high concentration of anti-CTLA-4 antibody into different groups.